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IDENTIFICATION AND DEVELOPMENT OF NOVEL ANTIMICROBIAL PEPTIDES AS ALTERNATIVES TO ANTIBIOTIC GROWTH PROMOTERS IN POULTRY

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Declaration

I declare that this thesis has been composed entirely by the candidate, Natasha Whenham. This work has not previously been submitted for a Doctor of Philosophy, a degree or any professional qualification. I have done all the work, unless acknowledged otherwise. All sources of information have been acknowledged.

Natasha Whenham

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Abstract

Poultry are vital to food security, with 60 billion chickens reared worldwide per annum and demand fast accelerating. For many years antibiotic growth promoters have been used to promote energy retention from the diet and control intestinal bacterial growth. Antibiotic use for prophylaxis or growth-promotion in farmed animals is prohibited under EU Directives due to human health concerns, but a pressing need exists to maintain the efficiency of animal production by finding alternatives.

Antimicrobial peptides (AMPs), part of the innate immune system exist naturally in most species and could provide a vast array of potential therapeutics. Microbial resistance to AMPs is unlikely due to their relatively unspecific mode of action, their ability to target multiple sites within a cell and diverse immune-modulatory activities. The avian egg provides antimicrobial protection through many mechanisms including AMPs which are incorporated into the egg white by the hen. The ovodefensin family and ‘transiently expressed in neural precursors’ (TENP) have been identified as potential novel antimicrobials in egg white and therefore formed the basis of the peptide portfolio of this study.

TENP was first identified as having a role in neurological development but has since been shown to be an important egg component constituting ~0.1-0.5% of the total protein. *TENP* is conserved across avian species being found in chicken, turkey, duck and zebra finch. Its homology with the bacterial permeability-increasing family of innate immune genes suggests it may contribute to antimicrobial function in the egg. This study confirmed that expression of *TENP* is confined to the albumen forming region of the oviduct in adult hens and is under gonadal steroid control, typical of an oviduct and egg specific gene.

The ovodefensin family are β defensin related antimicrobial peptides thought to be restricted to the albumen producing region of the avian oviduct. This study identified twenty five novel ovodefensin members through genome analysis, expanding the ovodefensin family to include reptiles for the first time. Phylogenetic analysis showed a unique example of the evolution of a cysteine spacing motif alongside traditional sequence evolution. The expression of eight ovodefensins was shown to be oviduct specific supporting the hypothesis that ovodefensins evolved to protect the egg. Antimicrobial activity for three ovodefensins from chicken and duck was investigated against gram negative organisms *E. coli* and *Salmonella* including pathogenic strains as well as a gram positive organism, *S. aureus*, for the first time. The spectrum of activity varied greatly between peptides suggesting a link between structure and function.

Inclusion of recombinant ovodefensin peptides in the feed of chickens showed beneficial effects on the gut microbiome, metabolite profile and most crucially an increase in mean body weight. This demonstrates the potential of antimicrobial peptides as alternatives to antibiotic growth promoters in poultry.

Publications

Original peer reviewed papers

Whenham, N., Wilson, P. W., Bain, M. M., Stevenson, L. & Dunn, I. C. 2014. Comparative biology and expression of TENP, an egg protein related to the bacterial permeability-increasing family of proteins. *Gene*, 538, 99-108.

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Abbreviations

3D	Three dimensional
AGP	Antibiotic growth promoter
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
APEC	Avian pathogenic <i>E. coli</i>
ATP	Adenosine triphosphate
AvBD	Avian beta defensin
BLAST	Basic local alignment search tool
BLAT	BLAST-like alignment tool
BNF	Buffered-neutral formalin
bp	Base pair
BPI	Bactericidal/permeability-increasing
BPIL	Bactericidal/permeability-increasing-like
BSA	Bovine serum albumen
BT	BT-TAMUS 2032
CCDA	<i>Campylobacter</i> blood-free selective agar
cDNA	Complementary DNA
CETP	Cholesterol ester transfer protein
CFU	Colony forming units
cm	Centimetre
CO₂	Carbon dioxide
dBPS	Duck basic protein small
DC	Dendritic cell
DES	Diethylstilbestrol
dH₂O	Deionised water
DHPS	Dihydropteroate synthetase
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	European Commission
ED50	Median effective dose
EST	Expressed sequence tag
EU	European Union
FCR	Feed conversion ration
FOS	Fructooligosaccharide
g	Gram
gDNA	Genomic DNA
GIT	Gastrointestinal tract
H₂O	Water
H₂O₂	Hydrogen peroxide
H₂SO₄	Sulfuric acid
hBD	Human beta defensin
HCl	Hydrochloric acid

HDP	Host defence peptide
HH	Hamburger–Hamilton
HIER	Heat induced epitope retrieval
HNP	Human neutrophil peptides
HRP	Horseradish peroxidase
IBV	Infectious bronchitis virus
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
kDa	Kilodalton
kg	Kilogram
l	Litre
LB	Luria broth
LBP	Lipopolysaccharide binding proteins
LBR	Lamin beta receptor
LPLUNC	Long PLUNC
LPS	Lipopolysaccharide
LSD	Least significant difference
LT	Lipid transfer
m	Metre
M	Molar
MBW	Mean body weight
MDCK	Madin-Darby kidney cells
mg	Milligram
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
mM	Millimolar
MOS	Mannan oligosaccharide
mRNA	Messenger RNA
NaCl	Sodium chloride
Na₂CO₃	Sodium carbonate
Na₂HPO₄	Disodium hydrogen phosphate
Na₂S·9H₂O	Sodium sulfide nonahydrate
NCBI	National Center for Biotechnology Information
NE	Necrotic enteritis
ng	Nanogram
nm	Nanometre
NMR	Nuclear magnetic resonance
OPD	O-Phenylenediamine
OvoD	Ovodefensin
P	Phosphorus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell

PFU	Plaque forming units
PLTP	Phospholipid transfer protein
PLUNC	Palate, lung and nasal epithelial clone
PMN	Polymorphonuclear leukocytes
qPCR	Quantitative PCR
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNase	Ribonuclease
RP-HPLC	Reverse phase high-performance liquid chromatography
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RTD	Rhesus theta defensin
RT-qPCR	Reverse transcription quantitative PCR
s	Second
SCFA	Short chain fatty acid
sem	Standard error of the mean
SPLUNC	Short PLUNC
TBS	Tris-buffered saline
TBST	Tris-buffered saline/Tween-20
TENP	Transiently expressed in neural precursors
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UTP	Uridine triphosphate
UV	Ultraviolet
VFA	Volatile fatty acid
vol	Volume

Latin species and common names

<i>Gallus gallus</i>	Chicken
<i>Meleagris gallopavo</i>	Turkey
<i>Anas platyrhynchos</i>	Duck
<i>Taeniopygia guttata</i>	Zebra finch
<i>Cygnus atratus</i>	Black swan
<i>Columba livia</i>	Rock Pigeon
<i>Melopsittacus undulatus</i>	Budgerigar
<i>Ficedula albicollis</i>	Collared flycatcher
<i>Geospiza fortis</i>	Medium ground finch
<i>Chrysemys picta bellii</i>	Painted turtle
<i>Caretta caretta</i>	Loggerhead turtle
<i>Pelodiscus sinensis</i>	Chinese soft-shell turtle
<i>Anolis carolinensis</i>	Anole lizard
<i>Alligator mississippiensis</i>	American alligator

Greek letters

α	Alpha
β	Beta
θ	Theta
μ	Mu (micro)
χ	Chi

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Chapter 1

Introduction

1. Introduction

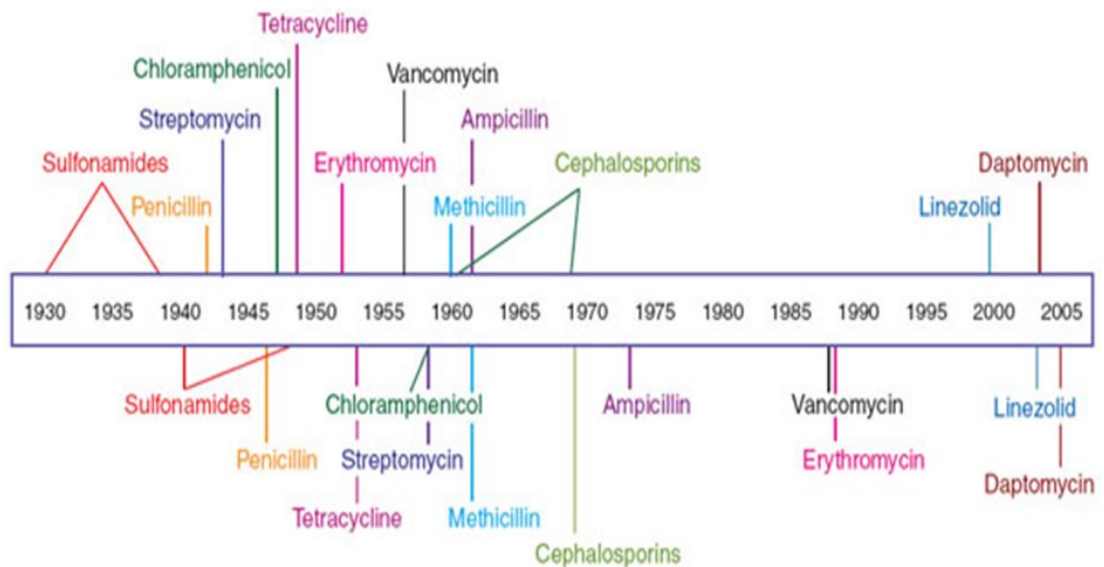
1.1 Poultry production and food security

The world food economy is being increasingly driven by food consumption trends and a dietary shift towards livestock products (Bruinsma, 2003). Within this food economy poultry represent a major protein source constituting around one third of the world's meat production. It is currently estimated that 55 billion birds are reared worldwide each year and demand is fast accelerating owing to population growth and rising affluence. It is predicted that production will need to increase by up to 60% to meet global demand by 2050 (FAO, 2013). Modern poultry have been bred to achieve high growth rates in a relatively short time period through improved feed conversion efficiency. In order to fulfil their maximum efficiency poultry need high quality feed ingredients, in particular cereals such as wheat (Willems et al., 2013). The inherent linkage of livestock production to the crop sector which supplies the feed stuffs for both poultry and man means that production of poultry affects the food economy as a whole and its efficiency is therefore intrinsically linked to food security (Commission of the European Communities et al., 1997). In addition to food security the way in which livestock is reared and the measures put in place to control disease affect the food safety of the final products. With around a million incidences of food poisoning annually in the UK at a cost of nearly £1.5 billion it is of great importance that food is raised not only efficiently but with safety of the final product in mind (FSA, 2011). Until recently this had been at least in part achieved by the use of antibiotics for growth promotion and disease prevention (Barton, 2000).

1.2 Antibiotics

Antibiotics were traditionally referred to as “chemical substances produced by various microorganisms, especially bacteria, fungi and actinomycetes, and having the capacity, in dilute solutions, to inhibit the growth of or to destroy bacteria and other microorganisms” (Lietman, 1986). This definition however does not allow the inclusion of substances that achieve the same antimicrobial activity but that are not produced by microorganisms. This includes compounds such as sulphonamides,

Antibiotic deployment



Antibiotic resistance observed

Figure 1.1: Antibiotic timeline. Diagrammatic representation of antibiotic introduction (depicted above the timeline) and the first observation of antibiotic resistance (depicted below the timeline) for the most commonly known antibiotics (Clatworthy et al., 2007).

man-made semisynthetic modifications of microbial products and chemically modified microbial products. It is therefore now more generally accepted that antibiotics are “a substance produced by or a semisynthetic substance derived from a microorganism, or a wholly synthetic substance able in dilute solution to inhibit or kill a microorganism” (Lietman, 1986). The key to antibiotics is selective toxicity; they must exhibit their antimicrobial effect without harming the host. It is therefore key that the processes which they affect are either non-existent in humans or differ significantly from the analogous function (Gale, 1960). There are many types of antibiotics based on molecular structure but the majority of these can be grouped into four categories; cell wall active agents, cell membrane active agents, protein synthesis inhibitors or enzyme interactors. With this in mind many of today’s antibiotics act on one of five essential bacterial processes: cell wall, DNA, RNA, protein or folate synthesis (Baron, 1996).

The first of the synthetic antibiotics were the sulphonamides, which interfere with bacterial folate (vitamin B₉) synthesis through competitive inhibition of dihydropteroate synthetase (DHPS) (Henry, 1943, Aminov, 2010). Then, a discovery by Alexander Fleming in 1928 led to what most people would associate with the start of the antibiotic era; the use of the first microbially derived antibiotic in humans, penicillin in 1943. This was followed by a boom in the discovery of antibiotic compounds throughout the mid 1900’s (Figure 1.1) (Aminov, 2010).

However the rate of discovery of novel antimicrobials has drastically dwindled and antibiotic resistance is quickly observed when new compounds are introduced (Clatworthy et al., 2007). This void in the development of new antibiotic compounds and the increasing levels of antibiotic resistance saw the issue recommended for inclusion in the national risk register at the same level as terrorism, with the premise that man is fast approaching an ‘antibiotic resistance era’ (DoH, 2013). An excerpt from Fleming’s Nobel Prize lecture in 1945 demonstrates that this is not a wholly surprising phenomenon.

“But I would like to sound one note of warning. Penicillin is to all intents and purposes non-poisonous so there is no need to worry about giving an overdose and poisoning the patient. There may be a danger, though, in underdosage. It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body”.

There are three classic mechanisms by which bacteria develop resistance to an antibiotic. The first is by developing an enzyme which is able to directly inactivate the antibiotic and stop it functioning. The second is to modify the target site of the antibiotic so that it can no longer bind and therefore loses function. Within this category we can also include the ability to produce decoy target sites which the antibiotic harmlessly binds to. Lastly the bacteria are able to reduce the permeability of their membranes so that the antibiotics cannot enter and in some cases they have developed efflux pumps to remove antibiotics that make it into the cell. The first two mechanisms of resistance can often be transferred from bacteria to bacteria whereas the third tends to be clone specific (Silva, 1996).

1.2.1 Antibiotics for growth promotion

One traditional use for antibiotics, other than for human or veterinary medicine, is the use for growth promotion in animal livestock. The relationship between a healthy gut microbial population and resistance against enteric pathogens is extremely important and maintaining homeostasis is vital in ensuring optimal animal health (Lee et al., 2010). For many years antibiotic growth promoters (AGPs) have been used as a method of controlling intestinal bacterial growth and ensuring optimal animal health in poultry (Bedford, 2000). When used as growth promoters antibiotics are usually administered in low dosages in the feed or water, in this way they have been shown to improve not only efficiency and performance but also uniformity of a flock (Singer and Hofacre, 2006).

Studies have shown that the use of AGPs with germ-free birds has no performance benefits; this suggests that they work through modification of intestinal microbiota

and have no physiological interaction with the bird itself (Bedford, 2000). The mechanisms by which AGPs confer this performance enhancement are not clearly understood however the main theories are as follows. Firstly it is generally accepted that the intestinal microbial load competes with the host for nutrients thus utilising a significant proportion of dietary energy, therefore a reduction in microbial numbers increases the amount of nutrient available to the bird (Apajalahti, 2005). Additionally a reduction in microbial numbers can result in the suppression of specific microbial by-products such as ammonia which is known to increase the rate of enterocyte replacement (Rinttila and Apajalahti, 2013). Energy which is then used to maintain gut integrity must be diverted from elsewhere, often at the expense of growth. In some cases bacteria produce an immune response, this requires energy that may otherwise have contributed to growth, and further more immune responses can lead to appetite depression, again ultimately affecting growth (Bedford, 2000). It should also be noted that as well as generally reducing bacterial load AGPs have been shown to reduce the level of harmful pathogens, in particular the gram positive organism *Costridium perfringens* (Stutz et al., 1983) which is responsible for the enteric disease necrotic enteritis as discussed in the next section.

1.2.2 Antibiotic withdrawal

There has been much debate about the crisis of antibiotic resistance in human pathogens and the source of the problem. Conflicting reports have been issued regarding the link between antibiotic usage in animals and resistance in human pathogens; although all generally emphasise a need for greater control in antibiotic usage across all sectors (Barton, 2000). Nonetheless, there is mounting evidence that antibiotic resistant bacteria can transfer to man from animals resulting in the establishment of a community reservoir of resistance genes (van den Bogaard and Stobberingh, 1999). Therefore the decision was made to remove antibiotic growth promoters from monogastric diets in the EU.

When the homeostasis of the gastrointestinal tract is disturbed through factors such as dietary changes, antibiotic usage or the introduction of novel microorganism it can

result in digestive disorder or disease (Lee et al., 2010). Digestive disorders such as dysbacteriosis occur when normally beneficial bacteria begin to grow unchecked and this overgrowth results in lost productivity through reduced nutrient availability for the host and gut inflammation (Teirlinck et al., 2011). The withdrawal of antibiotic growth promoters from poultry diets in 1999 has resulted in an increase in the incidence of enteric diseases (Bedford, 2000). It has been suggested that enteric disease can predispose an animal to colonisation and eventual infection of pathogenic organisms such as *Campylobacter* and *Salmonella* which are known to be significant causative agents of food poisoning (Choct, 2009).

A major outcome of antibiotic withdrawal in poultry has been an increase in the incidence of necrotic enteritis (Bedford, 2000). This poses a significant economic strain on the poultry industry due to the symptomatic problems of reduced growth, poor food conversion efficiency and in severe cases mortality (Choct, 2009). Necrotic enteritis is estimated to cost the poultry industry globally almost US\$ 2 billion each year (Dahiya et al., 2006). Necrotic enteritis (NE) was first described in broiler chickens by Parish in 1961 and is now the most common and financially devastating disease that modern poultry farmers have to contend with (Dahiya et al., 2006). The gram positive bacteria *Clostridium perfringens* is the main organism responsible, the enterotoxin it produces can be particularly problematic when the gut flora are still being established in young poultry and pigs (McDevitt et al., 2006). Symptoms of this potentially fatal disease include the sudden onset of diarrhoea and mucosal necrosis and are normally observed in chicks 2-6 weeks after hatching (Fukata et al., 1991). However an increase in mortality is not always immediately apparent and a reduction in growth rate is often the only observable consequence sometime after the event. The fact that this sub-clinical condition can go undetected, particularly in intensive production systems poses a real threat to the sustainability of the poultry industry as therapeutic administration of antibiotics tend to be applied too late (Porter, 1998, Cooper and Songer, 2009). Therefore there is a great need for alternative antimicrobial agents to control gut microflora and reduce the incidence of enteric disease without the need for reliance on therapeutic intervention.

1.3 Poultry digestive tract

The development of the gastrointestinal tract (GIT) begins *in ovo* and increases rapidly over the last four days of incubation with the villi developing in the last three (Shane, 2006). This rapid growth of the GIT continues post hatch with a peak at 6-10 days in broiler chicks. However the development of the gizzard and pancreas is much slower which limits nutrient digestibility and therefore the yolk sac provides the essential nutrients in this period for GIT growth and maintenance (Shane, 2006). It has been clearly demonstrated that immediate access to food post hatch beneficially improves nutrient digestibility by promoting earlier secretion of enzymes from the pancreas and brush border of the intestinal mucosa (Uni and Ferket, 2004). The development of villi also increase nutrient absorption through increasing the surface area of the GIT and immune protection is improved by mucin production which contains innate immune molecules such as defensins from hatch (Shane, 2006).

The basic digestive organs of poultry consist of the beak, oral cavity, oesophagus, crop, stomach (proventriculus and gizzard), small intestine, paired caeca, large intestine (colon) and terminate at the cloaca (Figure 1.2) (Shane, 2006).

1.3.1 Oesophagus and crop

The oesophagus is a highly dilatable musculo-membranous tube extending from the beak region to the stomach. Little digestion takes place here but the secretion of mucous from oesophageal glands aids the movement of food to the stomach. The crop is a specialised region of the oesophagus and as such very little digestion takes place here also. The crop acts as a food reservoir and regulates digesta transit time via emptying mechanisms which are dependent on capacity, digestion in the stomach and food particle size. When the stomach is empty peristaltic waves in the

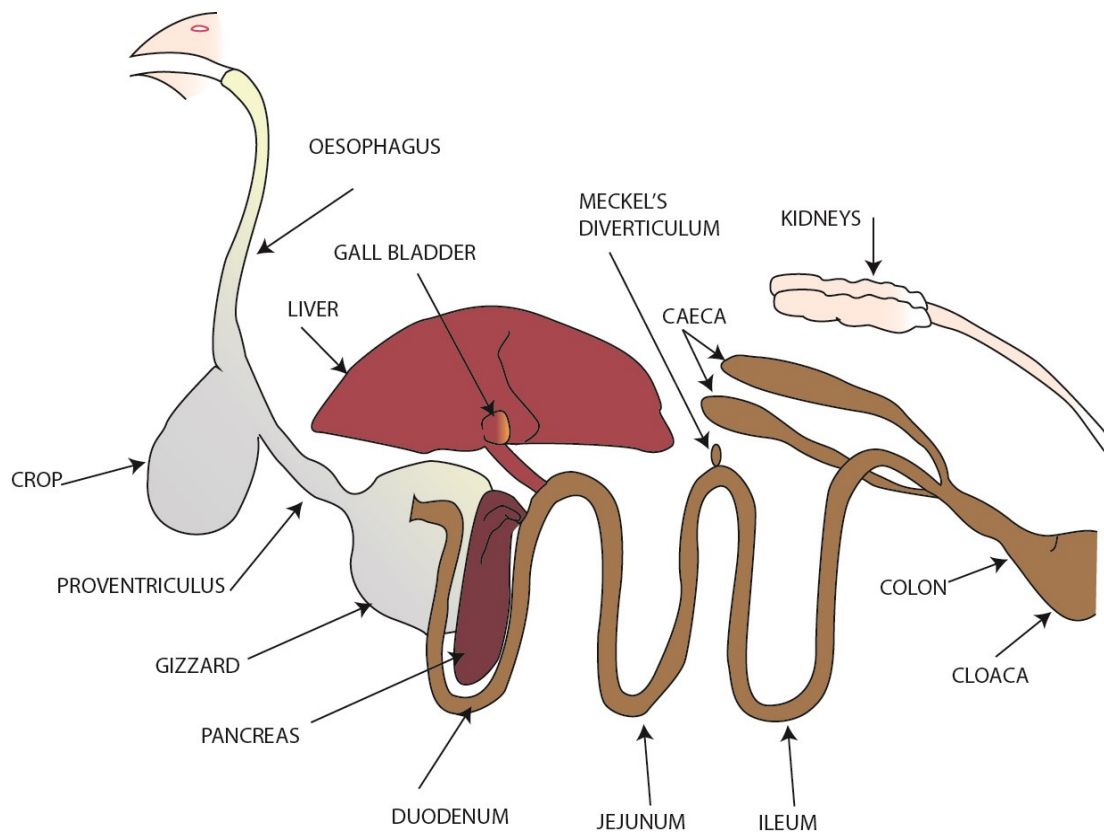


Figure 1.2: Diagrammatic representative of the chicken digestive tract.
(<http://www.poultryhub.org/physiology/body-systems/digestive-system/>)

oesophagus help the transport of food to the stomach (King and McLelland, 1975, Shane, 2006).

1.3.2 Stomach

The avian stomach, which includes the chicken, generally consists of two parts, the glandular part known as the proventriculus and the muscular region known as the gizzard (see Figure 1.2). The proventriculus is essentially an enlargement at the end of the oesophagus which produces pepsin and hydrochloric acid (HCl). However very little chemical digestion takes place in the proventriculus due to the rapid digesta transit time and gastric proteolysis takes place in the muscular gizzard. The muscular stomach (gizzard) consists of two muscle parts covered by a thick epithelium which physically grind the digesta; this is sometimes aided by the swallowing of grit or pebbles. True protein digestion starts in this region with activated pepsin from the proventriculus. Gastric contractions enable the digesta to be moved in both directions to allow further pepsin/HCl treatment in the proventriculus if required (King and McLelland, 1975, Shane, 2006), indeed retrograde peristalsis is an important mechanism in chickens, much more so than in mammals (Jimenez et al., 1994).

1.3.3 Small intestine

The small intestine connects the stomach to the caeca/large intestine and is the main site of digestion and nutrient absorption; it can be divided into three regions. In chickens the duodenum is approximately 20 cm long and forms a distinct loop. The distinction between the jejunum and ileum is less obvious and the Meckel's diverticulum (yolk stalk) is normally used as a landmark to separate them, the combined length of this region is approximately 120 cm (King and McLelland, 1975, Shane, 2006).

Within the small intestine digestion is enzymatic. Enzymes for the digestion of carbohydrates, lipids and proteins e.g. amylase, lipase and trypsin are produced in the pancreas and transported to the lumen of the small intestine. Bile is secreted into the

duodenum from the gall bladder which helps to neutralise acid from the proventriculus enabling other digestive breakdown. Bile also plays a key role in lipid digestion by emulsifying lipids to allow lipase binding and activity. Additionally the intestinal wall of chickens contains mucosal projections called villi which are covered in enterocytes as well as some mucous producing goblet cells. Enterocytes are covered with microvilli which form the 'brush border' lining the small intestine, this is where absorption of digested nutrients takes place and is the site of terminal carbohydrate digestion through brush border enzymes such as maltase and sucrose. Smaller peptides are also broken down to amino acids here through the enzymatic activities of amino peptidase and dipeptidases from the pancreas (Shane, 2006).

1.3.4 Caeca

Caeca arise at the junction of the ileum and large intestine; in chickens they are paired and are particularly large (up to 20 cm long). Although they have little digestive function caeca contain symbiotic micro-organisms which are able to digest cellulose and fibre to volatile fatty acids (VFAs) such as acetate, propionate and butyrate. The concentration of VFAs, particularly acetate, in the caeca is high and VFAs are therefore passively absorbed by cells. The caeca also plays an important role in amino acid absorption (King and McLelland, 1975, Shane, 2006).

1.3.5 Large intestine and cloaca

The large intestine (colon) is a short (~7 cm), straight region terminating in the cloaca. The large intestine allows the absorption of some water and electrolytes however undigested feed is quickly excreted and the absence of villi mean that little digestive function takes place here (King and McLelland, 1975, Shane, 2006).

1.4 Poultry intestinal microflora

The intestinal microbiota of an animal species has undergone co-evolution with its host and has enormous metabolic potential, affecting both the nutrition and health of

the host (Rinttila and Apajalahti, 2013). Each individual gut compartment of the GIT has its own unique physiochemical attributes and microbial community (Dethlefsen et al., 2007); the number of microorganisms in the GIT vastly outnumbered the host cells reaching 10^{11} - 10^{12} /g in the distal intestine (Savage, 1977, Whitman et al., 1998). With this in mind it is unsurprising that intestinal microflora affects intestinal health and ultimately, even in the absence of disease, animal production.

The intestinal microflora of a broiler chicken starts developing from hatch and initially consists of microbes from the egg shell which originate from the mother and also that of the surrounding environment. Exposure to this post-hatch inoculum is critical in the establishment of the intestinal gut community and plays a key role in shaping the immune system of the bird thus effecting intestinal microbiota over its lifetime (Apajalahti et al., 2004).

The bacterial density of the chicken GIT develops rapidly from hatch with the proximal and distal regions of the intestine reaching 10^8 and 10^{10} cells/g digesta respectively just one day post hatch in a broiler chick. Maximal density is reached within one week of hatch and remains relatively stable in healthy birds (Apajalahti et al., 2004). Facultative aerobes such as *Enterobacteriaceae* and *Streptococcus* are the initial colonisers of the GIT and become established in the small intestine and caeca within two-four days post hatch. After seven days *Lactobacillus* is the predominate organism of the small intestine (Barnes et al., 1972, Lu et al., 2003). The oxygen consumption of these facultative aerobic species results in a more reducing condition in the lower intestine allowing subsequent colonisation by obligate anaerobes and the caeca becomes predominately colonised by *E. coli* and *Bacteroides* (Barnes et al., 1972, Gong et al., 2002, Wise and Siragusa, 2007).

During the development of a mature GIT the bacterial flora undergo a transition towards the profile we associate with an adult bird. The upper GIT or small intestine consisting of the duodenum, jejunum and ileum is where the majority of digestion takes place and therefore the majority of nutrient absorption also (Renner, 1965). Due to rapid passage of digesta and low pH the bacterial counts in the duodenum and

jejunum are low, however these increase dramatically towards the distal end of the ileum where enzymatic activity is reduced (Apajalahti et al., 2004, Rinttila and Apajalahti, 2013). The most dominant species in this region are *Lactobacilli* which make up approximately 80-90% of the commensal bacteria; the remaining organisms are mainly *Enterobacteria* and *Enterococci*. As these commensals are able to utilise the same readily fermentable nutrients as the host this is where the main dietary competition between microflora and host takes place (Apajalahti, 2005). Normally the host can recover part of the energy lost to the commensal microflora through utilising bacterial fermentation end products (Rinttila and Apajalahti, 2013).

Intestinal microbial fermentation principally produces short-chain fatty acids (SCFA) including acetate, butyrate, lactate and propionate as end products (Topping and Clifton, 2001, Hooper et al., 2002, Bjerrum et al., 2006). SCFA production in the caeca of the chicken produces readily used energy sources for the host and lowers the pH of the intestinal environment which is thought to inhibit acid-sensitive pathogenic bacteria such as *Enterobacteriaceae* (Apajalahti, 2005). Of all the SCFAs produced by microbial fermentation, butyrate is thought to be one of the most beneficial as it is the preferred energy source of enterocytes and can regulate cell proliferation and differentiation within the intestinal mucosa resulting in an improved intestinal barrier (Le Blay et al., 2000, Fukunaga et al., 2003). Butyrate is produced by many microbial species found in the caeca; these include many *Clostridial* clusters from the *Firmicutes* phylum as well as *Lachnospiraceae* family members such as *Roseburia spp* and *Eubacterium rectale* (Rinttila and Apajalahti, 2013). However these species are particularly sensitive to changes in carbohydrate intake which in turn can affect butyrate levels in the lower GIT (Duncan et al., 2007). Butyrate has also been shown to increase endogenous defensin expression in the intestine thus contributing to the immune protection of the GIT (Sunkara et al., 2011).

The vast amount of research into the function of the gut microbiota has led to the insight that most interactions between the microbiota and the host are either commensal with the microbes benefiting without harm to the host or, mutualistic in which both the host and microbes benefit (Dethlefsen et al., 2007). The beneficial

relationship with commensal intestinal microbiota is well known: they aid digestion and synthesis of beneficial dietary compounds; they play a role in gastrointestinal development and epithelial proliferation, inflammatory immune responses and the energy metabolism of the host (Noverr and Huffnagel, 2004, Guarner, 2006, Forder et al., 2007, Klasing, 2007). Commensal bacteria can also synthesise vitamins and they fill an environmental niche that may otherwise be colonised by harmful enteric pathogens; in return they are provided with a constant supply of nutrients and secure growth conditions (Savage, 1977).

1.5 Current research into antibiotic alternatives

There are many ways in which we are able to influence the intestinal microbiota that do not involve the use of traditional antibiotics. It is likely that the immediate response to the withdrawal of prophylactic antibiotic usage will be an increase in the therapeutic usage, and indeed this has been seen in countries such as Denmark (DANMAP, 2010). Ironically this will likely increase the incidence of the emergence of resistant microorganisms and therefore many suggestions have been put forward for an antibiotic-free alternative to gut modulation (Bedford, 2000). Many of these strategies can only partially compensate for, rather than replace AGPs completely and it may ultimately be necessary to employ more than one strategy. Although any strategy should of course be combined with good hygiene management and consideration of diet composition. Broadly speaking most alternative strategies aim to either limit the nutrient availability to intestinal bacteria, promote the dominance of beneficial commensals or improve the immunity of the animal to disease (Bedford, 2000).

1.5.1 Probiotics

Direct-fed microbials or probiotics have been previously defined as “live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance” (Fuller, 1999). It is suggested that probiotics when fed early in life can influence the intestinal environment and favour the establishment of beneficial bacteria thus reducing the likelihood of pathogenic colonisation (Bedford,

2000). The proposed mechanisms of probiotics include: (a) competitive exclusion and antagonism of pathogens through the maintenance of beneficial commensals, (b) altering metabolism decreasing bacterial ammonia production and enzyme activity whilst increasing digestive enzyme activity, (c) improving feed intake and digestion, (d) neutralising enterotoxins and (e) stimulating the immune system (Dahiya et al., 2006). There are a number of possible mechanisms which may be responsible for the competitive exclusion of pathogens. These include competition for mucosal binding sites and nutrients or production of inhibitory substances such as volatile fatty acids or bacteriocins which are antibacterial for pathogenic bacteria (Tannock, 1997).

Probiotics are broadly categorised as ‘undefined’ or ‘defined’ preparations. Undefined probiotics contain preparations of ‘normal’ avian gut microbiota originating from healthy adult individuals which are free from specific pathogenic microorganisms. Defined preparations on the other hand comprise of a single or very small number of characterised bacterial strains (Dahiya et al., 2006). Preparations are normally fed orally to newly hatched chicks in order to prevent colonisation by pathogens in the rearing environment (Nurmi et al., 1992, Nisbet, 1998).

Interestingly a number of studies have reported that undefined preparations have a beneficial effect on the incidence of necrotic enteritis including reduced mortality and caecal colonisation. For example, Craven *et al.* (1999) demonstrated a reduction in *C. perfringens* colonisation and subsequent reduction in the incidence of necrotic enteritis. Another field study found that the use of undefined microflora preparations delayed the intestinal proliferation of *C. perfringens* and the appearance of necrotic lesions (Kaldhusdal et al., 2001).

1.5.2 Prebiotics

Another potential approach to modulate the chicken gut microbiome is the dietary supplementation of prebiotics. Prebiotics are indigestible feed ingredients that selectively stimulate the growth or activity of resident beneficial bacteria thus proving advantageous for the host (Gibson and Roberfroid, 1995). To be classed as a

prebiotic the ingredient must not be hydrolysed or absorbed in the small intestine and must be a selective substrate for beneficial bacteria in the large intestine thus promoting their growth and activity (Collins and Gibson, 1999). Most prebiotics are polysaccharides including fructooligosaccharides (FOS) and lactulose although it has been suggested that some oligosaccharides such as mannan oligosaccharides (MOS) may exhibit some prebiotic activity (Dahiya et al., 2006).

1.5.3 Organic acids

Organic acids are already widely used in Europe in the poultry industry for antimicrobial protection of raw ingredients and finished feed against pathogenic organisms such as *Salmonella* (Dahiya et al., 2006). Depending on the concentration and type of organic acid used it can either act as a carbon or energy source for bacteria or as an inhibitory agent (Cherrington et al., 1991). Although widely used the mode of action of organic acids is not fully understood however the basic principle is that they are able to penetrate the cell wall of certain types of bacteria and disrupt the normal physiology (Dahiya et al., 2006). Lambert and Stratford propose that on exposure to the internal pH of the bacteria the acid will dissociate releasing H⁺ and anions. This will decrease the internal pH which is not tolerated by many bacteria as they utilise a H⁺-ATPase pump to bring the pH back to a normal level. This requires a large energy expenditure which can lead to reduced growth or even death of the bacteria. In addition to this it is thought that the anionic part of the acid also gets trapped within the bacteria and is eventually toxic (Lambert and Stratford, 1999).

The performance enhancement recorded in broilers is dependant on dose, type of acid used and whether the acid is included in the diet or drinking water (Patten and Waldroup, 1988). Propionic acid has been shown to successfully reduce the level of *E. coli* in the intestinal tract of broilers when included in the diet (Izat et al., 1990) and was effective at alleviating enteritis in turkey poults (Roy et al., 2002). It has also been suggested that organic acid inclusion in drinking water can eliminate

Campylobacter potentially reducing *Campylobacter* infection in chicks (Chaveerach et al., 2004).

1.5.4 Herbs, spices and essential oils

Herbs, spices and their essential oils have been used as pharmaceuticals in alternative medicine and natural remedies for many years. Herbs have been shown to have antiviral and antioxidative properties and have been found to enhance antimicrobial activity as well as stimulate the endocrine and immune system (Dahiya et al., 2006). They are said to promote a higher metabolic status and several botanical ingredients are reported to have beneficial effects on the gut microbiome (Rao and Nigam, 1970, Besra et al., 2002). Essential oils are reported to stimulate digestive enzymes and many have shown antibacterial activity *in vitro* including essential oil of clove which killed greater than 99% of several bacterial strains including *S. aureus* and *E. coli* (Briozzo et al., 1989). However, although there are many studies reporting the effectiveness of essential oils *in vitro* there have been few *in vivo* trials to test this in an animal model and it may be that the essential oils would need to be greatly concentrated to work in such a system (Dahiya et al., 2006). However some promising research by Mitsch et al. (2004) found that specific blends of essential oils were able to reduce the proliferation of *C. perfringens* in the broiler intestine which may in turn reduce the incidence of necrotic enteritis.

1.5.5 Feed enzymes

Feed enzymes have been considered as a means for improving animal performance and health in antibiotic growth promoter-free broiler diets. Enzymes benefit the host through increasing the rate of diet digestibility and improving nutrient availability; additionally this improvement also affects the substrate quality and quantity available to the intestinal microflora (Bedford, 2000). For example if the in-feed inclusion of an enzyme results in less undigested starch and protein in the anterior small intestine then the result is less nutrient availability for the microflora. As a consequence, microbial populations are reduced and with this potentially the risk of disease (Bedford, 2000). The effect of feed enzymes is also more apparent in diets high in

indigestible components, for example the use of xylanases and β -glucanases significantly increase utilisation of diets high in xylans and β -glucans (Wu and Ravindran, 2004). It is difficult to determine if an improvement in animal performance is due to the improved digestibility of the diet or a reduction in microbial populations. However it is clear that the effect of feed enzymes is much more pronounced when there is a significant residential microbial population and as such are likely to be more effective in the absence of AGPs (Bedford, 2000).

1.5.6 Bacteriophages

Bacteriophages are viruses that are capable of entering bacterial cells and using the bacteria's own metabolic machinery to replicate before utilising cell lysis to kill the host and release new phages into the environment (Joerger, 2003). These viruses have received much attention as potential alternatives to antibiotics due to their highly specific activity towards bacterial replication (Joerger, 2003). In mice bacteriophages were successfully used to treat vancomycin-resistant *Enterococcus faecium* infection and it has also been suggested that bacteriophages could be used to control necrotic enteritis in broilers as they are also effective against *C. perfringens* (Biswas et al., 2002). Additionally Sklar and Joerger observed that phage-treated chickens had lower counts of *Salmonella enteritidis* in the caeca than untreated birds (Sklar and Joerger, 2001). The use of bacteriophages has not only been suggested as a replacement for AGPs but they may also be useful in microbial control of feed and food meaning they could be effective along the whole production pathway (Dahiya et al., 2006). However phage therapy against intestinal bacteria may prove more difficult than uses outside of the bird as there is likely to be little support from the immune system. Therefore any bacteria with resistance mechanisms to phage attack may be able to multiply favourably and affect the delicate balance of the intestinal microflora (Joerger, 2003).

1.5.7 Nutrition: diet formulation and ingredient selection

The importance of nutrition in the poultry industry is well documented and it has been demonstrated that both the physical and chemical attributes of the diet can not

only modify the intestinal microbiome but also affect the integrity of the intestinal epithelium (Langhout et al., 1999, Apajalahti et al., 2001). The quality of the raw ingredients used in the feed has a direct effect on bird performance; higher quality materials have a greater host digestibility which results in reduced substrate availability for bacterial growth. Higher quality ingredients also tend to contain less antinutritional factors such as lectins that can damage gut epithelial tissue (Bedford, 2000). Diet composition also affects the intestinal microbiome; a study documenting broiler microbiomes by Apajalahti in 2004 reported that increased numbers of *Enterococcus* were recorded when a feeding corn and sorghum based diets whereas barley increased the number of *Lactobacillus*. Apajalahti also documented that the growth of *Lactococcus* and *Escherichia* were promoted when an oat based diet was fed and rye increased the *Streptococcus* populations (Apajalahti, 2004). An increase in *Lactobaccili* was also observed when poultry were fed a diet containing linseed (Alzueta et al., 2003) and numerous studies have reported the reduced incidence of necrotic enteritis in poultry fed corn based diets due to reduced viscosity when compared to those fed diets based of wheat, oats or rye (Dahiya et al., 2006). Additionally the use of whole cereal grain in the diet has also been shown to result in more efficient digestion as whole grains stimulate gizzard development resulting in a digesta with a smaller average particle size (Svihus et al., 1997). Furthermore some nutrients are known to be potentiators or down regulators of the immune system (Korver and Klasing, 1997, Klasing, 1998) and suppression of inflammatory responses may prove beneficial to growth rate providing the bird is still able to illicit an effective immune response against disease (Bedford, 2000). One such dietary compound of interest in immune modulation is linoleic acid (Cook, 1998). Another consideration when formulating the diet is the level of protein and amino acid supply. Undigested protein acts as a food source for putrefactive organisms in the lower gut which can be detrimental to health; it is therefore beneficial to limit excess protein in the feed (Bedford, 2000, Drew et al., 2004).

1.5.8 Host defence peptides

Host defence peptides (HDPs) are naturally occurring components of the innate immune system and play a role in the antimicrobial protection of the host (see 1.3.1) (Zasloff, 2002, Espinoza et al., 2003, Ganz, 2003). They have recently received a lot of attention as potential alternatives to antibiotics due to their direct ability to kill bacteria, particularly as topical agents for microbial infection (Hancock and Lehrer, 1998, Hancock and Sahl, 2006). For example a hybrid of cecropin (moth) and melittin (bee venom) was effective at treating *Pseudomonas aeruginosa* in rabbit eye infections (NosBarbera et al., 1997). Additionally nisin, a broad spectrum bacteriocidin was able to reduce *S. aureus*, *Klebsiella pneumoniae* and *E. coli* numbers when applied topically to cow teat skin (Sears et al., 1992). In addition to their direct killing activities some HDPs are also potent immunomodulators and have the ability to dampen down potentially harmful pro-inflammatory responses which make them interesting candidates for an entirely new class of therapeutic agents (Hancock and Sahl, 2006, Bowdish et al., 2006). Indeed some trials have shown promising results for treating systemic infections with host defence peptides. For example two α -helical peptides derived from silk moth cecropin (MBI-27 and MBI-28) were effective at treating a *P. aeruginosa* peritoneal infections (Gough et al., 1996) and a pig derived β -sheet-protegrin showed promise in treating methicillin-resistant *S. aureus* and *P. aeruginosa* infections (Steinberg et al., 1997). Although much of the therapeutic interest in HDPs has focussed on infection and disease they may have potential uses in the poultry industry linked to the withdrawal of antibiotic growth promoters (Bedford, 2000). There has been little research done to this end and the work that has been carried out has focussed on protection to disease challenge rather than modulation of the intestinal microbiota. One particular study of interest fed the cationic peptide BT-TAMUS 2032 (BT) from the soil bacterium *Brevibacillus texasporus* to broiler chickens for four days post hatch. In this study BT peptide resulted in protection from extra-intestinal challenge by *Salmonella enterica* Enteritidis and up-regulated functional efficiency of heterophils (Kogut et al., 2010). A repeat of this study confirmed the previous finding and demonstrated

that there was also increased functional efficiency of monocytes when compared to broilers fed a control diet (Kogut et al., 2012). This work gives an exciting insight into the possibilities of the in-feed application of HDPs although whether peptides such as these, or indeed those from the host itself are able to replicate the growth-enhancing effects of AGPs has yet to be elucidated.

1.6 The innate immune system

The innate immune system is responsible for providing the host with the mechanisms and machinery it requires for protection against invading organisms. The first line of defence is a physical one, epithelial cells provide an anatomical barrier that prevents most invading organisms from gaining access to the host. Any organisms that have adhered to this surface are removed when the surface is shed during desquamation. Also in the gastrointestinal and respiratory tract ciliary movement helps to move adhering pathogens and mucous traps the infectious agents (Ryu et al., 2010). In the gut the natural microflora of the host helps to prevent the colonisation of pathogenic microbes by secreting toxic substances to kill invading organisms. They also compete with pathogenic microbes for nutrients and space which helps prevent colonisation of unfavourable organisms and in turn prevent the associated diseases (Sadowska and Rozalska, 2008).

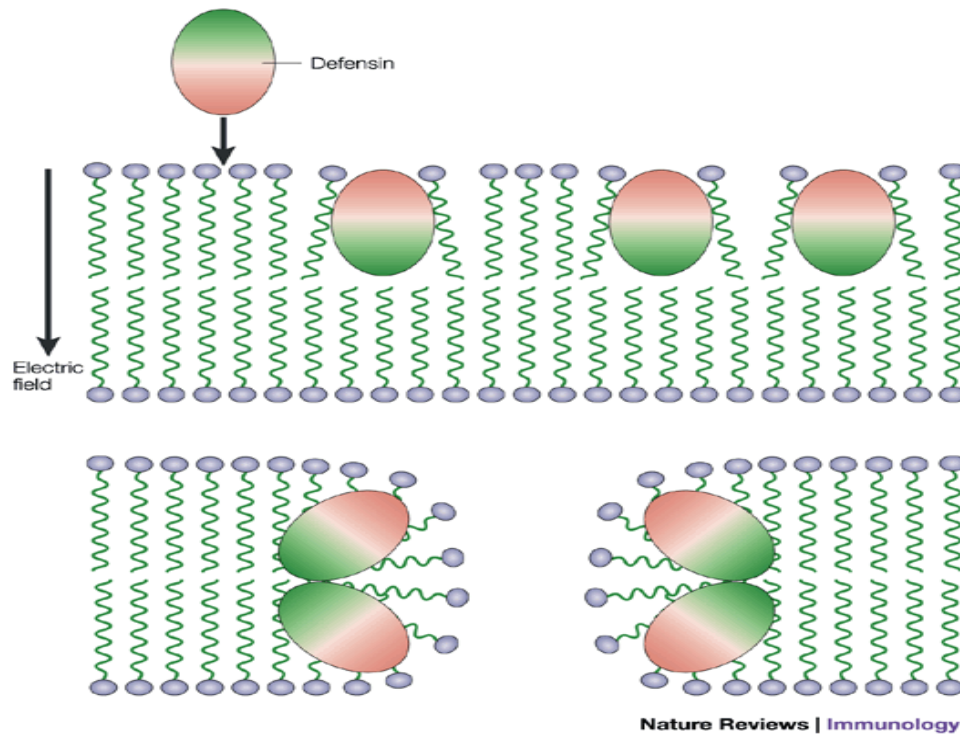
The innate immune system also produces chemical factors such as cytokines which recruit immune cells to the site of infection. Cytokines mediate an inflammatory response which provides a physical barrier to prevent the spread of infection whilst injured cells are repaired and infectious agents are cleared from the system. The innate immune system also activates the adaptive immune system through antigen presentation by macrophages and dendritic cells to T-cells so a 'specific' immune response to that pathogen can take place. The 'non-specific' innate immune system is evolutionarily older than the adaptive immune system and is also responsible for producing antimicrobial agents which can directly kill invading organisms (Janeway, 1993).

Two particular families of innate immune peptides/proteins were identified as candidates for this study; host defence peptides (see 1.6.1), more specifically defensins (1.6.1.1) and the lipid transfer/lipopolysaccharide binding family of proteins (see 1.6.2).

1.6.1 Host defence peptides

As part of the innate immune system epithelia cells secrete peptides which kill or slow the growth of invading microbes (Zasloff, 2002, Espinoza et al., 2003, Ganz, 2003). Host defence peptides (HDPs) are a diverse group of small, cationic peptides found in a wide variety of organisms (Cuperus et al., 2013). Originally known as antimicrobial peptides (AMPs) their principal mode of action is dependent on their interaction with the bacterial cell membrane, although it is suggested that their lethal mode of action involves disruption to the cytoplasmic membrane (Figure 1.3) (Hancock and Rozek, 2002). This interaction is thought to be driven by an electrostatic attraction between the positively charged peptide and negatively charged components on the outer bacterial envelope. In gram positive bacteria these negative components are thought to be lipoteichoic acids and in gram negative the phosphate groups within the lipopolysaccharides (LPS). The result of these interactions is either the disruption of the cell membrane or the translocation of the peptide across the membrane into the cytoplasm to interact with internal targets without causing major disruption to the membrane. There have been many proposed models for the exact events that happen at the cell membrane but they all lead to one of three outcomes: formation of a transient channel, micellarisation of the membrane or translocation across the membrane (Jenssen et al., 2006).

In addition to their direct antimicrobial activity it is becoming increasingly accepted that HDPs exhibit an immunomodulatory effect (Figure 1.4). It is suggested that this effect has the dual function of being able to stimulate immune function in response to potentially harmful pathogens to prevent infection, as well as suppressing the pro-inflammatory response to avoid excessive inflammation. Some antimicrobial



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Figure 1.3: Proposed model of defensin pore formation. The amphipathic nature of most defensins (shown as ovals) allows them to interact with microbial membranes. The positively charged region of the peptide (pink) interacts electrostatically with the negatively charged phospholipid headgroups of the bacterial membrane and the defensin is pulled into the membrane, this is in part due to the hydrophobic nature of the other amino acid side region (green). As the defensins accumulate in the membrane they move into another arrangement (shown in the lower panel) and pores are created (Ganz, 2003).

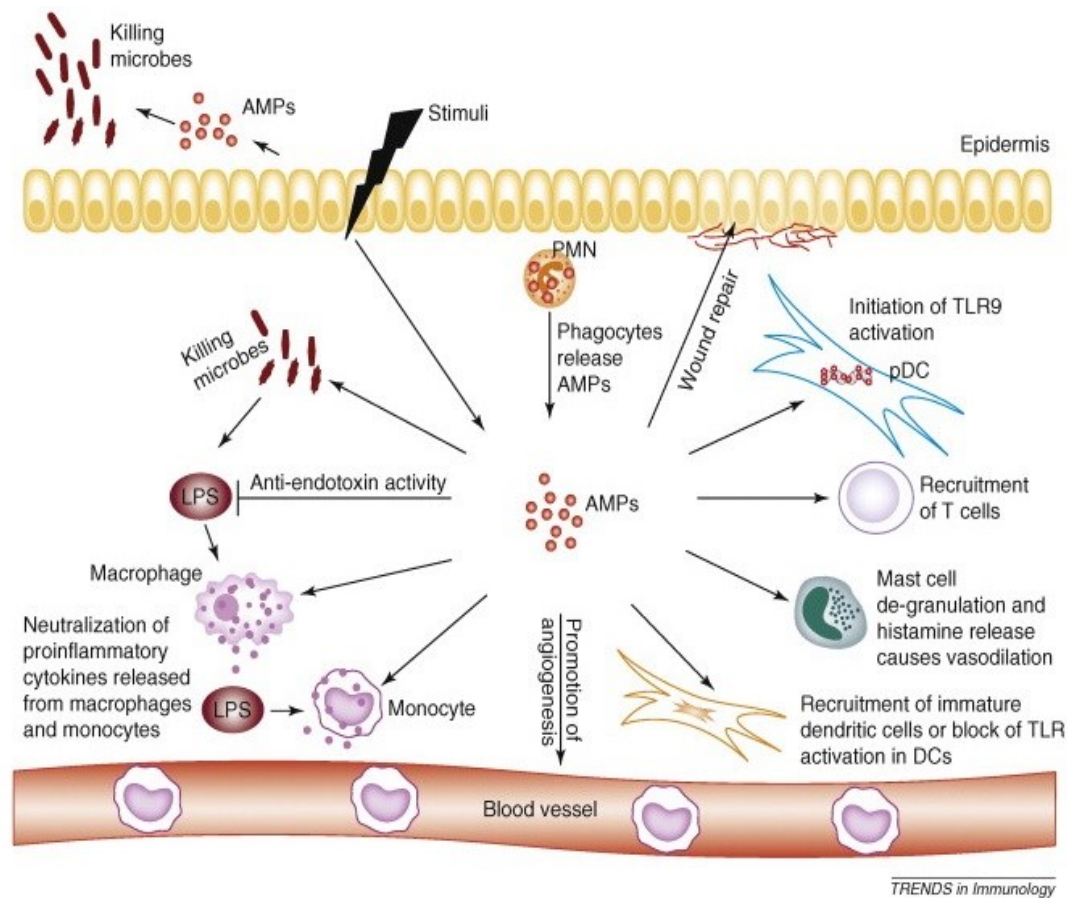


Figure 1.4: Multiple immunomodulatory functions of antimicrobial peptides in host defence. Host defence peptides (HDPs), otherwise known as antimicrobial peptides (AMPs) have multiple functions in in host defence. AMP activity includes the induction of responses in immune cells such as monocytes and macrophages, alteration of gene expression in host cells and induction of chemokine and cytokine production (Lai and Gallo, 2009).

peptides also have the ability to synergise with cytokines in order to modify their immunomodulatory activity (Auvynet and Rosenstein, 2009).

The chemotactic activity of HDPs are particularly effective for leukocytes and despite an overlap in activity of different HDPs they are able to complement each other in order to direct effector cells to the site of inflammation and modulate local immune response by organising the appearance of different immune cells in different situations (Lai and Gallo, 2009). For example human cathelicidin LL-37 is effective in attracting neutrophils, monocytes and T-cells (Lai and Gallo, 2009) whereas human β -defensin 2 (hBD2) is able to recruit mast cells (Niyonsaba et al., 2002a). Certain α -defensins such as human neutrophil peptides (HNP) 1 and 2 can increase local densities of monocytes (Territo et al., 1989) whilst hBD3 and hBD4 are both chemotactic for monocytes and macrophages (Yang et al., 2002). The result of all these effects is the promotion of antigen presentation to stimulate the clonal expansion of B and T-lymphocytes and to mount an adaptive response which then contributes to the clearance of microbes through phagocytosis (Auvynet and Rosenstein, 2009). As well as these chemo-attractant activities some HDPs indirectly promote chemotaxis through the induction of chemokine secretion, for example interleukin-8 (IL-8) release in lung epithelial cell lines can be induced by LL-37 (Scott et al., 2002, Tjabringa et al., 2003).

HDPs are also able to function as anti-inflammatory molecules with many processes for this activity having been observed. Some HDPs such as LL-37 have been observed to neutralise the endotoxin effects of LPS and in doing so limit the extent of inflammation (Scott et al., 2002). HDPs also have the ability to abolish the expression of pro-inflammatory molecules such as IL-6 and TNF- α , and LL-37 has been shown to modulate the toll-like receptor (TLR) response by interacting with TLR ligands through an LPS-binding mechanism (Mookherjee et al., 2006). In addition to this they are able to selectively permeabilise the membranes of apoptotic leukocytes through a mechanism similar to their direct antimicrobial mode of action discussed previously (Bjorstad et al., 2009).

A combination of all the functions of HDPs allows both the triggering of the immune response and the termination of the immune response through the elimination of immune cells and a shift in balance between pro-inflammatory and anti-inflammatory effects.

1.6.1.1 Defensins

Defensins are a family of evolutionarily related cationic host defence peptides with a characteristic framework of 6 cysteines linked by 3 disulphide bonds. There are two main subfamilies of defensins, the beta defensins and alpha defensins. Beta defensins are characterised by a beta pleated sheet structure. Both subfamilies consist of a triple stranded beta sheet and characteristic defensin-fold yet differ in the length of peptide residues between cysteines and the arrangement of disulphide bonds between each of the cysteine residues. Another structurally distinct subfamily of defensins, the theta defensins also exist, these form a cyclic peptide and the cysteine pairing differs from both α and β defensins (Figure 1.5) (Ganz, 2003, Xiao et al., 2004). It has recently been suggested that the folded structures produced by the disulphide bonds may not be important in the antimicrobial activity of the peptide and that their activity is in fact increased when the peptide is linearised. However, linear mutants of human beta defensin 1 with the cysteines replaced with arginine show a loss of activity, particularly those without the three cysteine residues at the carboxyl terminal (Schroeder et al., 2011). This could suggest that the availability of the cysteines may be an important factor in the peptides activity. The folded form of the peptide may therefore provide the host with the ability to produce and store these peptides in an inactive form to be activated through breakage of the disulphide bonds when the host is challenged by an infectious agent.

In birds both α and θ defensins appear to be absent, however several β defensins (or gallinacins) have been isolated suggesting they are the oldest of the defensin families (van Dijk et al., 2008). To date 14 avian β defensins have been identified in the chicken genome, all being encoded on chromosome 3 (Lynn et al., 2007). Phylogenetic analysis of vertebrate defensins shows clustering of chicken and

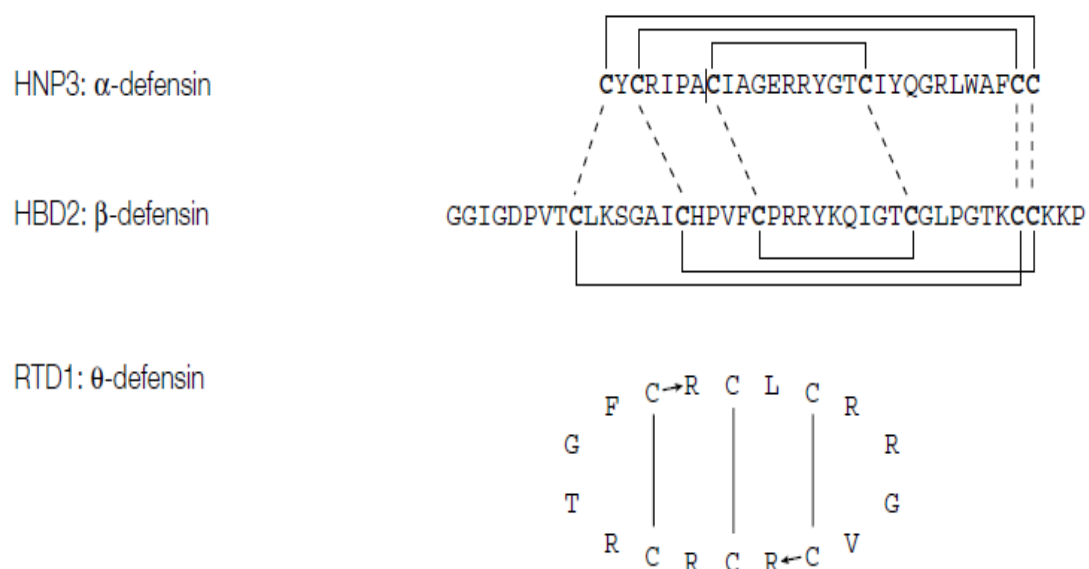


Figure 1.5: Sequences and disulphide pairing of cysteines of α , β and θ defensins. Corresponding cysteines between the α and β defensins are indicated by a dotted line and disulphide bonds are represented by a solid line. In α -defensins the six cysteines are linked in a 1-6, 2-4, 3-5 formation whereas in β -defensins the pattern is 1-5, 2-4, 3-6. Circular θ -defensins do not have a free N or C-terminus.

mammalian β defensins suggesting that this family arose before birds and mammals diverged (Xiao et al., 2004). Defensin-like sequences can also be found in plants and insects where they are also involved in the innate antimicrobial defence of the host (Raj and Dentino, 2002). The variety of multicellular organisms capable of producing defensin peptides means that defensins can be considered as an ancient first line of defence against pathogenic organisms.

Defensins are synthesised in granulocytes such as neutrophils in mammals (Duits et al., 2002) and heterophils in poultry (Evans et al., 1995), or secreted by epithelial cells (Zhao et al., 2001). Avian β defensins (AvBD) are stored in heterophils as a tripartite prepropeptide sequence with a NH_2 -terminal precursor signal sequence, a neutral or basic propiece and a mature cationic peptide. In α defensins the negative charge of the propiece usually neutralises the positive charge of the mature peptide thus preventing premature interactions with the epithelial membrane (Ganz, 2003). However the propeptide of AvBD1 is not negatively charged and AvBD2 only has one negative charge and it is therefore unlikely that they are able to neutralise the mature peptide in this way. As such it is likely that there is another mechanism responsible for preventing premature activity of chicken β defensins (Brockus et al., 1998). Defensins are widely distributed across a variety of tissues and are either constitutively expressed or produced in response to microbial infection. For example in the chicken AvBD1 and AvBD2 are present in heterophil granules (Harmon, 1998) as well as tissues including the lung and testis (van Dijk et al., 2008) whilst AvBD3 is constitutively expressed in tissues such as the tongue, skin and oesophagus (Zhao et al., 2001). AvBD8 has so far only been detected in the liver and gall bladder and AvBD7 appears to be found mostly in the bone marrow (van Dijk et al., 2008). The diverse expression of defensins suggests a generalised protective role of these peptides as part of the innate immune system.

Antimicrobial activity of defensins has been observed against bacteria, fungi, parasites and viruses and under optimal conditions such as low ionic strength is observed at concentrations as low as 1-10 $\mu\text{g/ml}$ (Ganz, 2003). Avian β defensins 1

and 2 were both found to be greater than 90% effective against *Escherichia coli* and *Listeria monocytogenes* at 16 µg/ml (Evans et al., 1995).

As with all host defence peptides described previously defensin activity is primarily associated with their ability to depolarise and permeabilise microbial membranes. This is achieved through electrostatic interactions with negatively charged components of the outer membrane e.g. LPS in gram negative bacteria and teichoic acid in gram positive bacteria (Wimley et al., 1994). Defensins are also involved in immune modulation through processes such as chemotactic activity as discussed previously (1.3.1).

1.6.2 Lipid transfer/lipopolysaccharide binding proteins

The human lipid transfer/lipopolysaccharide binding proteins (LT/LBP) are a gene family; the membership of this family is based on sequence homology. They are structurally related proteins that are capable of binding phospholipids and lipopolysaccharides, albeit with differing affinities. The founding members of this gene family are bacterial permeability-increasing protein (BPI), lipopolysaccharide binding protein (LBP), phospholipid transfer protein (PLTP) and cholesterol ester transfer protein (CETP). All of these proteins are thought to arise from a common ancestor due to their amino acid similarity and intron/exon organisation (Mulero et al., 2002).

BPI is a ~50kDa, boomerang shaped, cationic protein consisting of two domains, BPI1 and BPI2 which have a very similar folded structure (Figure 1.6). These domains both contain a non-polar pocket which is capable of binding phospholipids. The BPI1 domain is the N-terminal half of the molecule and is responsible for mediating LPS and bacterial binding, antibacterial cytotoxicity and the neutralisation of endotoxins. The C-terminal (BPI2 domain) on the other hand is responsible for opsonisation and mediating an immune response (Schultz et al., 2001). More recent additions to this family are the bacterial permeability-increasing like-proteins BPIL1, BPIL2 and BPIL3. BPIL1 and BPIL3 form a cluster with BPI on chicken

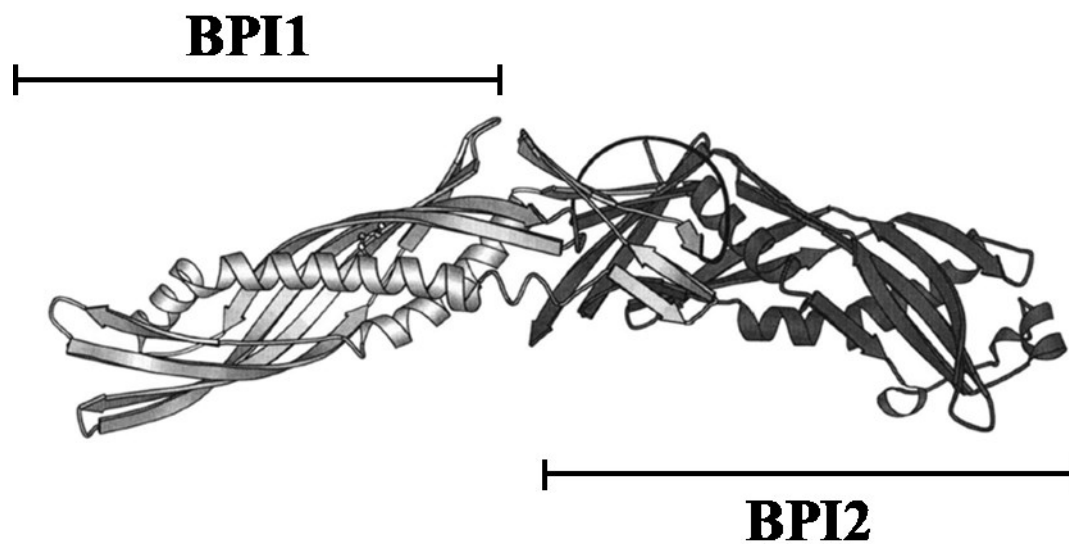


Figure 1.6: A ribbon diagram of human bacterial permeability-increasing protein (BPI). The N-terminal domain (BPI1) is white, and the C-terminal domain (BPI2) is grey (Beamer et al, 1998).

chromosome 20 whereas BPIL2 maps to chromosome 22. All three BPIL molecules share a highly conserved intron/exon organisation with BPI (Mulero et al., 2002).

1.7 The chicken oviduct

In chickens the reproductive system and in particular the egg is a large and varied source of antimicrobials (Kovacs-Nolan et al., 2005). This therefore seemed a good starting point for identifying novel antimicrobial candidates for study in this PhD thesis.

The female chicken reproductive system consists of the ovary and oviduct (Figure 1.7). Follicles are produced in the ovary and contain an ovum or yolk, which if reaching maturity will go on to be incorporated in an egg. A mature follicle passes into the infundibulum, the start of the oviduct, where fertilisation of the ovum by the spermatozoa can occur. From here it passes through to the magnum (or ampulla) where egg albumen (egg white) is secreted before passing through to the isthmus where the shell membrane is deposited. It is in the magnum that gallin, an ovodefensin is most highly expressed. Next the egg passes through to the shell gland (or uterus) where the egg shell and pigmentation is deposited before finally entering the vagina where it is positioned ready for lay (King and McLelland, 1975, Jonchere et al., 2010). In a non-laying bird the oviduct is short and relatively small in diameter, however, when the birds' oviduct becomes active during egg-laying the oviduct grows and can become up to 70-80 cm in length (Dougherty and Sanders, 2005).

1.7.1 The egg

The egg must provide an embryo with the nutrients it needs to grow as well as protection from pathogens to ensure it survives to hatch. An egg's antimicrobial defence can be considered as two major mechanisms; physical and chemical (Wellman-Labadie et al., 2007).

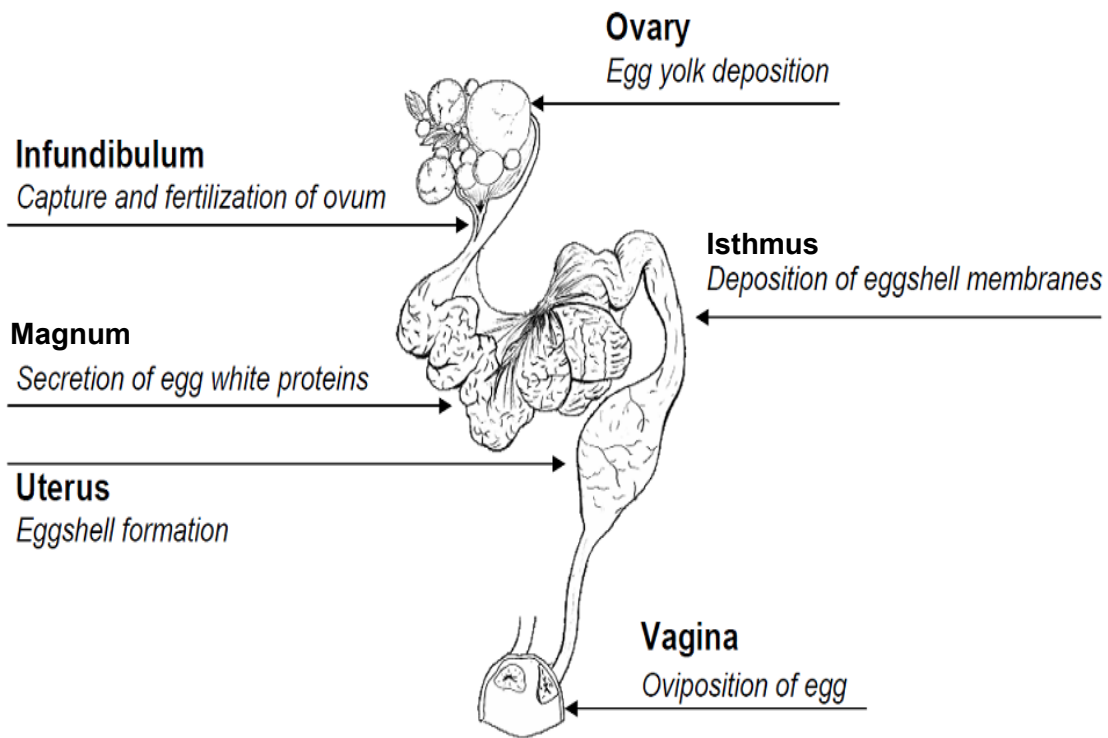


Figure 1.7: Diagrammatic representation of the avian oviduct (adapted from (Bréque et al., 2003)).

1.7.1.1 Physical defence

The shell and cuticle provide the first line of physical defence and acts as a barrier to microbial penetration; however the shell contains thousands of pores for allowing gaseous exchange which can be a route of entry to microbes. The shell membrane is composed of a network of fibres which are thought to act as a microbial filter to impede bacterial penetration. Although intact shell and the shell membranes offer physical protection it is not completely impenetrable and once bacteria have crossed this barrier the viscosity of albumen acts as a final physical hindrance in reaching the nutrient rich yolk (Nys et al., 2011).

1.7.1.2 Chemical defence

The relative alkalinity of egg albumin (pH 9-10) makes it inhospitable to many bacteria helping to prevent contamination; in addition to this albumen proteins provide antimicrobial protection through a variety of mechanisms. Some of the best known antimicrobial albumen proteins are lysozyme, ovotransferrin and avidin (Nys et al., 2011). It was suggested by Fleming in 1922 that lysozyme was able to cause bacterial cell lysis and it has since been demonstrated that this effect is achieved through catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, a major component of gram-positive bacterial cell walls (McKenzie and White, 1991). It is also suggested that lysozyme aids physical defence through forming a network with ovomucin that aids egg viscosity (Cotterill and Winter, 1955). Ovotransferrin is a heat stable glycoprotein that can bind iron and therefore plays an important role in nutrient transfer to the embryo. However its ability to bind iron also makes ovotransferrin an important antimicrobial agent in egg white as through binding iron it makes it unavailable to bacteria thereby hindering survival. The binding capacity of ovotransferrin is greatly reduced at low pH meaning the alkalinity of the egg accentuates its activity (Giansanti et al., 2012). Similarly, avidin indirectly functions as an antimicrobial through forming an indigestible complex with biotin and preventing its utilisation by bacteria (Laitinen et al., 2002).

1.7.1.2.1 Ovodefensins

One candidate of interest in the egg was gallin, a 41 amino acid peptide belonging to a particular group of avian defensins (see 1.3.1.1); the ovodefensins. This group of defensins are expressed in the oviduct of widely divergent bird species and it is thought that they play a role in protecting the growing embryo from pathogenic organisms whilst it is developing in the egg. Gallin has been shown to possess antimicrobial activity against *E.coli*, and three copies of the gene are expressed from 3 separate loci on chicken chromosome 3. A number of these ovodefensins have been identified in the genomes of the chicken, turkey, duck and zebra finch. Meleagrin has been described in the turkey, dBPS1 and 2 in the duck and taeniopygin 1 and 2 in the zebra finch (Gong et al., 2010). This study also observed another meleagrin-like gene in the turkey genome and a potential additional copy of taeniopygin 1 in the zebra finch genome. These extra copies may be an adaptation to increase levels of expression in the egg white as previously suggested for gallin. Interestingly the 3D structure of chicken ovodefensin gallin has recently been solved which confirmed the presence of the three-stranded antiparallel β -sheet observed in all classical beta defensins confirming its classification as a beta defensin (Herve et al., 2014). However, gallin contains an additional short two-stranded β -sheet (Figure 1.8) (Herve et al., 2014), this five-stranded arrangement supports the hypothesis that gallin, and presumably the other ovodefensins, form a structurally distinct sub-family of β -defensins.

1.7.1.2.2 Transiently expressed in neural precursors (TENP)

Another egg protein of interest is the protein ‘transiently expressed in neural precursors’ (TENP); it has been found to show homology with the BPI antimicrobial peptide family, in particular BPIL1 (Mulero et al., 2002). It was first described as a transiently expressed gene during neurogenesis in the chicken (Yan and Wang, 1998) and has since been identified during proteomic studies of the chicken egg white. It has been proposed that its function is associated with the innate defence of eggs

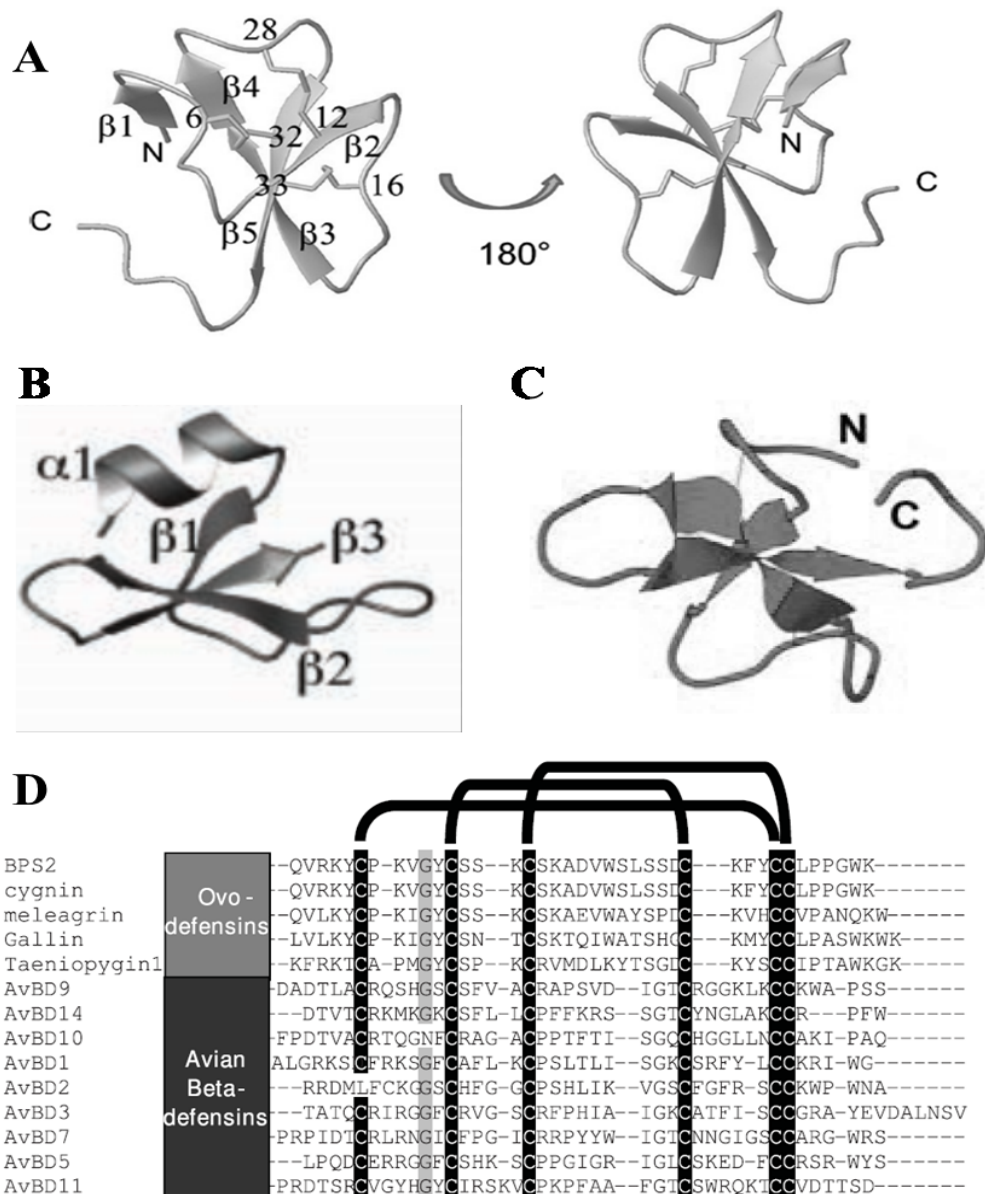


Figure 1.8: Comparison of defensin 3D structure and alignment of avian ovoidensins with classical avian β -defensins. The 3D structure of ovoidensin gallin (A) (Herve et al., 2014), human β -defensin 1 (B) (Bauer et al., 2001) and avian β -defensin 2 (C) (Zhang and Sunkara, 2014) are shown. Gallin contains an additional two β -sheets (labelled β 1-5) (A) when compared to both classical mammalian (B) and avian β -defensins (C). A CLUSTALW alignment between ovoidensins and classical avian β -defensins is shown in D: from (Gong et al., 2010). Conserved cysteines are highlighted in black with the black bars representing cysteine bonds; a conserved glycine is highlighted in grey. The difference in inter-cysteine spacing between ovoidensins and classical avian β -defensins is clearly apparent and gaps in the alignment are shown by a '- '.

against pathogens (Guerin-Dubiard et al., 2006, Mann, 2007, D'Ambrosio et al., 2008), however its proposed function is based on its relationship to the LT/LBP family and antimicrobial activity has yet to be experimentally proven.

1.8 Therapeutic potential of ovodefensins

Antimicrobial peptides could present an interesting alternative to prophylactic antibiotics if used as a feed additive due to their potential for rapid, broad spectrum bactericidal activity and low predisposition for resistance development (Marr et al., 2006). They are naturally occurring in the host cells of almost all species as part of the innate defence against invading organisms and could therefore provide a vast array of potential therapeutics. Cationic HDPs selectively choose bacterial cells due to their affinity for the highly negatively charged cell membranes. This makes the likelihood of interactions with eukaryotic cells, in which the cell surface consists of predominately uncharged lipids, low (Jenssen et al., 2006). Microbial resistance is unlikely due to their relatively unspecific electrostatic mode of uptake and their ability to target multiple sites within a cell. Resistance can generally not be directly selected for under laboratory conditions and requires multiple passages at sub minimum inhibitory concentration (MIC) levels as opposed to antibiotics which generally induce resistance after only a few passages at sub-MIC levels (Marr et al., 2006). Despite the risk of resistance being comparably less than traditional antibiotics some instances of resistance within specific strains of bacteria have been reported. For example some bacteria such as particular *Yersinia* strains are able to utilise efflux transporter systems to pump cationic antimicrobial peptides out of the cell (Bengoechea and Skurnik, 2000). Other bacteria such as certain strains of *Klebsiella*, *Streptococcus* and *Pseudomonas* produce anionic capsule polysaccharides which block the bactericidal activity of AMPs by binding them, thereby reducing the amount of peptides reaching the bacterial surface (Llobet et al., 2008). Both of these resistance mechanisms revolve around the required direct association between cationic HDP and the bacterial cell. However as well as their direct interaction with micro-organisms HDPs play a role in mediating the immune response (Hancock and Scott, 2000), therefore the eradication of pathogens may still be possible due to this

indirect effect on the invading organism (Oyston et al., 2009). In addition to the eradication of pathogens, prophylactic HDP use may make it possible to reduce the inflammatory response of the host without reducing immune protection; this would allow the redistribution of energy and potentially avoid the reduction in growth often observed during infection. In order to increase the activity of a given dose, combinations of HDPs could be formulated for a synergistic effect. This could either be to reduce the required minimum dosage or to broaden the spectrum of activity.

There are of course some limitations and critical needs that need to be addressed for the development of HDPs as alternative AGPs, or indeed as a therapeutic in any application. Firstly in order to create a range of products it is necessary to be able to discover a large number of antimicrobial peptides to identify those with different spectrums of bactericidal activity and immunomodulatory properties. Testing a wide variety of peptides could also aid in the design of synthetic peptides with improved function, stability or lability. However the single largest issue in peptide therapeutics is arguably the high cost of solid-phase peptide production and to date efforts to test and develop a large number of variants has largely been limited by this expense. The high cost of peptide production will indeed also limit the potential clinical targets to which these molecules can be applied. Potential toxicity of HDPs would also need to be addressed as well as understanding the real efficacy of the peptides in the context of animal models as opposed to the efficacy *in vitro* (Hancock and Lehrer, 1998, Hancock and Sahl, 2006). Recombinant systems such as the fungal system used in this PhD may provide the tools for cheaper production this allowing screening of a greater number of candidates which will aid the addressing of these limitations.

1.9 Assay selection

The limitations of testing naturally occurring antimicrobial peptides *in vitro* has long been under scrutiny and the potency of their activity is often directly affected by the conditions they are tested under (Schwab et al., 1999). Whether the peptide is tested under solid or liquid phase conditions or the absence/presence of salt, serum or proteins in the test conditions have all been shown to affect activity (Schwab et al.,

1999). There is also some debate on how relevant *in vitro* results are for inferring activity *in vivo*; in any instance the activity observed is specific to the conditions of the assay and may not reflect activity in different conditions. Before moving to testing *in vivo*, the studies in this PhD used an *ex vivo* gut model to evaluate promising candidates against a mixed community of microorganisms that directly resemble those conditions in which the peptide would need to work in terms of microbial microbiome structure, pH and metabolites.

1.10 Project aims

Antibiotic use has driven the evolution of transmissible antibiotic resistance that has reduced the effectiveness of human and veterinary medicines (Hancock, 1997). A recent ban under EU Directives of in-feed antibiotics used to promote energy retention from the diet and prevent microbial infections has led to production losses, increased incidence of disease and welfare concerns in the poultry industry (Bedford, 2000). Poultry are crucial to global food security and therefore a pressing need exists to maintain the efficiency of animal production by finding alternatives. One of the critical needs in realising the potential of alternative antimicrobial strategies was identified as the characterisation of a wide range of molecules and their properties (Hancock and Sahl, 2006). Antimicrobial peptides have been suggested as an alternative due to their potential for rapid, broad spectrum bactericidal activity and low predisposition for resistance development (Hancock, 1997). The large abundance of natural antimicrobials make the egg an invaluable reservoir of potential candidates (Kovacs-Nolan et al., 2005) particularly as the clinical application for this study relates to the food industry. Naturally derived egg products, as already widely consumed by humans, would offer a level of acceptability and therefore form the basis of the peptide portfolio for this PhD study.

The overall aim of this study was to identify novel antimicrobial peptides from the egg and characterise their biology, evolution and function for consideration as peptide feed additives.

The first phase of the project focused primarily on identification of novel candidate peptides. Available literature was used to prioritise peptides from the egg that were largely uncharacterised but had some supporting evidence of antimicrobial function or homology with known antimicrobials. Phase two involved characterisation of the biology of selected candidate genes, in particular evolutionary aspects, transcript investigation and expression analysis. Finally the most promising candidates were assessed for antimicrobial function, initially *in vitro* and in an *ex vivo* gut model but ultimately in an *in vivo* peptide feed trial.

The aim of the research described in chapters 3 and 4 was to determine if the expression of selected genes was restricted to the oviduct and therefore indicative of an egg specific protein. This was achieved through RT-qPCR analysis across a wide range of tissues and where possible was confirmed at protein level with immunohistochemistry. The purpose of these chapters was also to identify homologs of each gene to determine if they were avian specific or spanned other species to gain an insight on the drivers of evolution.

In chapter 5 selected candidates were evaluated for antimicrobial activity in an *in vitro* assay and one candidate was assessed under challenge conditions (pH and salt). The most promising candidates were selected for trial in an *ex vivo* gut model to determine activity in a more relevant setting and evaluate whether they could modulate whole gut microbiomes.

Finally chapter 6 evaluated the efficacy of two peptides in an *in vivo* peptide feed trial. Mean body weight was used as an indicator of growth performance and parameters such as intestinal microbiota and metabolites were used as correlates of good gut health. This trial resulted in improved growth and a reduction in known pathogenic bacteria such as *Clostridium perfringens* showing promise for the use of antimicrobial peptides as alternatives to antibiotic growth promoters.

Chapter 2

Materials and Methods

2.1. Reagents and solutions

All medium and buffer recipes are shown in Appendix 1.

2.2. Bioinformatic analysis

2.2.1. TENP

The TENP protein sequence (NCBI accession no. AF029841) was used to perform a TBLASTN search of the May 2006 release of the chicken EST database using the default parameters of the NCBI blast database (<http://blast.ncbi.nlm.nih.gov/>) to identify ESTs and sequences related to the locus. The Staden package (Staden, 1996) was used to build a consensus sequence using the ESTs available. This allowed a gap present in the genome build to be bridged and allowed a comparison to be made between the genomic, EST and published TENP mRNA sequences in order to address differences in sequence identity. Primer3(<http://frodo.wi.mit.edu/primer3/>) was used to design primers (Table 2.1) for re-sequencing across the length of the consensus sequence including a putative alternative transcriptional start site identified in the consensus sequence.

Signal P (Bendtsen et al., 2004) was used to assess the likelihood of a signal peptide produced by each translational start site. The protein sequence is submitted to the Signal P website in FASTA format using the eukaryotic setting; all other settings were used in default mode. A graphical output is produced showing the position of three different scores; C, S and Y on the sequence. The C score is the raw cleavage site score and indicates the most probable location of the first amino acid of the mature peptide; the score is highest at this point. The S score is the signal peptide score and is used to distinguish between positions within a signal peptide and other parts of the mature protein or proteins that do not have a signal peptide. The S score is therefore highest across the signal peptide and a loss in signal should coincide with a peak in the C score if a signal peptide is likely. Finally the Y score combines the C and S scores to give a likelihood of a signal peptide. This is required as it is possible to have multiple high C scores in any given sequence yet to determine a true signal peptide it is necessary to have an accompanying peak in the S score. In addition

signal P also provides two additional data points. The mean S value is the average S score across the whole of the predicted signal peptide and the D-score is an average of this mean S value and the maximal Y value. The D-score gives the overall prediction for the likelihood of a sequence containing a signal peptide. For non-secretory proteins all scores should be close to the negative target value of 0.1 (Petersen et al., 2011).

Exon contributions were estimated from genomic DNA using BDGP NNSPLICE version 0.9 (Reese et al., 1997) (Chapter 3, Table 3.2). Genomic sequences were submitted in FASTA format and default settings were used. Where a large number of potential splice sites were retrieved the minimal 5' and 3' splice site score were increased to 0.7. BDGP NNSPLICE analyses a sequence for donor and acceptor sites, the program only considers genes which conform to specific constraints with the consensus splice sites. For example GT for the donor and AG for the acceptor site, the outputs contain a score which indicates the likelihood of a particular splice site which is based on motifs in the surrounding sequence.

The duck genome database (*Anas platyrhynchos* – version 1) (<http://pre.ensembl.org>) and turkey genome database (*Meleagris gallopavo* – assembly UMD2) (<http://www.ensembl.org>) were searched using TBLASTN for potential homologues using the 629 amino acid mature TENP protein.

2.2.2. Ovodefensin

Available genome databases Ensembl (<http://www.ensembl.org>), PreEnsembl (<http://pre.ensembl.org>) and UCSC (<http://genome.ucsc.edu/>) were searched using TBLASTN and BlastP to locate potential homologs using the 41 amino acid mature peptide sequence of gallin (GenBank: CBE70283.1) and the previously published 39 amino acid mature peptide sequence of taeniopygin 2 (Gong et al., 2010). Further iterative searches were made with the homologues discovered. Protein database Uniprot (<http://www.uniprot.org/>) was also searched using BlastP to identify peptide sequences previously unidentified as ovodefensins. Primers (Table 2.2) were designed to ensure complete coverage of each ovodefensin's coding sequence.

Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence
TENP Exon1F1	AGGATGGGAACAGCAAACAG	TENP End1	ATCCTCCTTCTGCACCAAAA
TENP RTF	CACTGCTGGAGGAGCTGTT	TENP RTR	ACAACGTTGACGTCGGTGTA
D.TENP F3	AGACCATACGCAGAGGTGGT	D.TENP R3	AGGTTGCACAGGAGCAAGAC
D.TENP F8	AGGGAATCACCATGTCCTTG	D.TENP R10	TGATCTTCTGTGCCATGGTG
T.TENP F1	GTCTCAAAGCCACATGCAGA	T.TENP R1	GGTTTTCCACTGCTTTCACC
		TENP SP1	ATCTGGAGGACTTGCTTTCC
		TENP SP2	CCCATGTCTACGTGGAGGTC

Table 2.1 – Names and sequences of TENP primers used in the study.

Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence
Chicken OvoDB1 F/RTF	GTGCTCTTTGCTGTGCTCCT	Chicken OvoDB1 R/RTR	AGAGCTGCTCCTGCTCCAC
Turkey OvoDA1 F	TGGGCTAAGTTTCCCAGCTA	Turkey OvoDA1 R	AGTGACTGTGGCTGTCATCG
Turkey OvoDA1_2 F	AGCTCCTCTCCTCCAGCTCT	Turkey OvoDA1_2 R	TACTTTGCATGGGGTCAACA
Turkey OvoDB1 F/RTF	TGTGCTCCTGCTCTTTTCCT	Turkey OvoDB1 R/RTR	AGAGCCTCTCCTGCTCCACT
Duck OvoDA1 F	AGGGGGTGGCAGTTGAGTAG	Duck OvoDA1 R	GACGAAAGGCTGGAAAACAG
Duck OvoDB1 F	GAGATCCCCACCGTCCTC	Duck OvoDB1 R	TCAAAGCAACGAAACCGTCT
Zebra finch OvoDA1 F	AAATGGGGAAGAGCAATGG	Zebra finch OvoDA1 R	AATCGTGGGGACACCAGTAA
Zebra finch OvoDB1 F	GCTGTGGTCCTGGTGGATA	Zebra finch OvoDB1 R	TTGCTGCAAGCATCACTTTC
Chicken OvoDA1 RTF	CTCCAGCCTCGCTCACAC	Chicken OvoDA1 RTR	TTGAGAGGAGGGGATGACAC
Turkey OvoDA1/1_2 RTF	GCTGTCCTCCTGCTGGTCT	Turkey OvoDA1/1_2 RTR	CCTTGCAGTCGGAGGAGTAG
Duck OvoDA1 RTF	GCTACGGGTTTCCCCTTC	Duck OvoDA1 RTR	AGGTCCGGTACTGCTCCAG
Duck OvoDB1 RTF	GTGCTCCTGCTTTTCTCCAT	Duck OvoDB1 RTR	GCACGCAATGAACACAGC
Zebra finch OvoDA1 RTF	CCTTCCAGGCTATGGGAAGT	Zebra finch OvoDA1 RTR	GCAGTCGCCAGAGGTGTATT
Zebra finch OvoDB1 RTF	CGTTGTCTTTGCTGTTTTCCT	Zebra finch OvoDB1 RTR	GTGCTCCTCCCTCTCTCCTT
Chicken OvoDA1 SP5	ACACGTGCTCCAAGACACAG	Chicken OvoDA1 SP1	GCAGGCAGCAGTACATCTTG
		Chicken OvoDA1 SP2	GCCCAGATCTGTGTCTTGGA

Table 2.2 – Names and sequences of ovodefensin primers used in the study.

As described for TENP (2.2.1) Signal P (Bendtsen et al., 2004) was used to assess the likelihood of a signal peptide when a potential translational start site could be identified in the genome.

2.3. Phylogenetic analysis

2.3.1. TENP

A phylogram indicating the evolutionary history of TENP was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) from an alignment of the mature proteins. Included for comparison were human *LPLUNC2* (NCBI reference sequence no. NM_025227) and chicken *Ovocalyxin 36* (NCBI reference sequence no. NM_001030861). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The branch lengths are proportional to the evolutionary distances which were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). The units are the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The common name of the species is included with the protein names.

2.3.2. Ovodefensin

A distance matrix based on the amino acid sequence length between each of the cysteines and the conserved glycine residue was built in R for all known and newly discovered ovodefensins (Chapter 4, Table 4.1). Hierarchical clustering for each distance matrix was calculated using R (<http://www.r-project.org/>) which was then used to produce a cladogram of the phylogenetic relationships using the R ‘hclust’ function. Similarly cladograms based on spacing were produced separately for avian species and reptiles

In addition to this a cladogram was constructed using the Neighbour-Joining function (Saitou and Nei, 1987) in Mega5 with a bootstrap test of 1000 replicates (Felsenstein, 1985) of the core peptide sequence from the conserved glycine until the fourth cysteine residue inclusively for sequences where the number of amino acids within

this region was identical. These functions were again used in Mega5 to construct phylograms based on the whole mature peptide sequences for OvoDA family members and also OvoDB.

2.4. Birds and tissue collection

2.4.1. Broilers

Oviduct tissue (magnum, shell gland, isthmus, vagina), ovarian stroma and liver were collected from sexually mature broiler breeders (n=3). A diagrammatic representation of the region of the oviduct and descriptions of their function can be found in Chapter 1, Figure 1.7.

2.4.2. Layers

The following tissues were taken from sexually mature White Leghorn LSL hens (*Gallus gallus*) (Lohmann): Oviduct (magnum, shell gland, isthmus, vagina), ovarian stroma, crop, duodenal loop, gizzard, caeca, cloaca, lung, adrenals, cerebellum, retina, spleen, liver, kidney, and heart (n=4).

2.4.3. Turkeys

The following tissues were collected from sexually mature turkeys (Kelly Bronze, Scotland): Oviduct (magnum, isthmus, shell gland, vagina), ovarian stroma, oesophagus, crop, duodenal loop, gizzard, caeca, cloaca, lung, adrenals, cerebellum, tongue, spleen, breast muscle, skin, liver, kidney and heart (n=4).

2.4.4. Ducks

Tissue was collected from sexually mature Pekin ducks (*Anas platyrhynchos*) (Cherry Valley): Oviduct (magnum, shell gland, isthmus, vagina), ovarian stroma, crop, proventriculus, small intestine, duodenal loop, gizzard, large intestine, caeca, cloaca, gall bladder, lung, trachea, pituitary, adrenals, cerebellum, hypothalamus, tongue, spleen, breast muscle, liver, kidney and heart (n=3).

2.4.5. Zebra finches

Adult female zebra finches were supplied courtesy of Dr Karen Spencer, University of St Andrews, Scotland. Tissues collected were oviduct (magnum, isthmus shell gland), ovarian stroma, small intestine, duodenal loop, gizzard, lung, spleen, breast muscle, skin, liver, kidney and heart (n=5). Tissues for immunolocalisation studies (2.12) (magnum, isthmus, shell gland and breast muscle) were harvested from 5 of these laying hens birds post mortem and fixed in 10% buffered-neutral formalin (BNF) prior to being processed to paraffin wax.

2.4.6. Embryonic tissue

Brain, retina and heart tissue was taken from embryonic day 10 (E10) (n=6) and day 16 White Leghorn chickens (E16) (n=6). E10 and E16 correspond with Hamburger–Hamilton (HH) embryonic developmental stages HH36 and HH42 respectively.

2.4.7. Time of oviposition

Magnum tissue was obtained from sexually mature White Leghorn hens with an ovum at various positions in the oviduct; see Gong *et al.* (2010) for details. Briefly, magnum tissue was processed either when the egg was in the magnum (n=5), in the shell gland where the stage of calcification was determined by electron microscopy and recorded as early (n=8), mid (n=9) and late (n=10) calcification or during a pause day (n=11) when there was no evidence of ovulation.

The time of oviposition q-PCR on *Gallus gallus OvoDB1* was carried out by Maisarah Maidin as part of a summer placement project which I designed and supervised.

2.4.8. Effect of oviduct development

In domestic chickens the onset of incubation behaviour is characterised by the regression of the oviduct due to the withdrawal of gonadotrophic support (Dunn *et al.*, 1996). This natural phenomenon was exploited to determine if expression differed between in lay hens and those where the oviduct had regressed. Magnum

and shell gland tissue from both states was collected from hens of a Silkie x White Leghorn cross which readily show incubation behaviour, (n=11).

All tissues were rapidly dissected using sterile instruments and placed in RNA free Eppendorf tubes. After dissection tissue was either frozen in liquid nitrogen and directly stored at -80°C or placed in RNA later (Ambion, Applied Biosystems, Warrington, UK) and stored at 4°C overnight before storage at -80°C. Samples weighed no more than 100 mg.

2.4.9. Administration of steroid hormones

The administration of steroid hormones was adapted from a method described previously (Kunnas et al., 1992). Three week old ISA brown chicks (n=60) were given an intramuscular injection of 0.5mg diethylstilbestrol (DES) in 0.5 ml propylene glycol daily for seven days (primary stimulation) and then split into two groups. Following the primary DES treatment there was a period of withdrawal from DES for 12 days in group one (non-primed) birds (n=30) followed by a single injection of progesterone (20 mg/kg) (n=10), oestradiol (10 mg/kg) (n=10) or vehicle (propylene glycol; 1 ml/kg) (n=10). In the second group (primed) the birds (n=30) were re-stimulated daily for two days with DES after a withdrawal period of 10 days followed by a single injection of progesterone (n=10), oestradiol (n=10) or vehicle (n=10) as already detailed for the non-primed birds. All chicks were killed 12-16 hours after the single injection; magnum tissue was removed and immediately frozen in liquid nitrogen then stored at -80°C.

This experiment had been carried out previously by those named in the above paper (Kunnas et al., 1992); RNA was kindly provided for reverse transcription and subsequent q-PCR analysis.

2.5. RNA processing

2.5.1. RNA purification

Soft tissues (magnum, shell gland, isthmus, lung, cerebellum, spleen, liver, kidney and embryonic) were homogenised in Lysing matrix D tubes (Qbiogene-Alexis Ltd.

Nottingham, UK) containing Ultraspec II total RNA isolation reagent (AMS Biotechnology, Oxon, UK) using a FastPrep FP120 homogeniser (Qbiogene-Alexis Ltd. Nottingham, UK). All other tissues were homogenised in Ultraspec II total RNA isolation reagent (AMS Biotechnology, Oxon, UK) using a Ultraturrax homogeniser (IKA[®]-Werke GmbH & Co. KG, Germany). Samples were homogenised in 1ml of UltraSpec II solution and were kept chilled on ice throughout the homogenisation period.

Samples were then processed as per the Ultraspec protocol which utilises a phenol/chloroform solution to isolate RNA in the aqueous phase, this is then separated by centrifugation. The quantity and purity of the isolated RNA was checked using a nanodrop (Thermo Scientific, Wilmington, USA).

2.5.2. Reverse transcription

Total RNA (1 µg) was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, UK) according to the manufacturer's protocol. Before reverse transcription took place all samples were treated with RQ1 RNase-Free DNase (Promega, Madison, USA) as per manufacturer's instructions to remove potential residual genomic DNA contamination left over from the RNA purification.

2.6. Polymerase chain reaction (PCR)

2.6.1. Primer design

Primers were designed with Primer3 (<http://frodo.wi.mit.edu/primer3/>) using the default settings and all primer pairs were tested using the UCSC Genome Browser Gateway (<http://genome.ucsc.edu/>) *in silico* PCR tool to check specificity and ensure they did not amplify other regions of the genome.

2.6.2. PCR conditions and product purification

PCR was carried out using Faststart Taq (Roche, UK) according to the manufacturer's protocol. Amplification was performed using the following conditions: an initial denaturation at 95°C for 4 minutes, followed by 40 cycles of 30s at 95°C, 30s at 58°C and 30s at 72°C, followed by an extension of 7 minutes at

72°C. All products were separated by 2% agarose-gel electrophoresis and visualised using SYBR Safe DNA gel stain (Invitrogen, UK). Bands were excised from the gel and the cDNA purified from the matrix using a Qiaex II Gel Extraction Kit (Qiagen, UK). The Qiagen kit solubilises agarose to release the DNA from the matrix; this is then bound to silica gel particles under high salt concentrations. Dissociation of the DNA from the silica gel particles is achieved through subsequent washes in a low salt solution.

The purified PCR fragments were sequenced with their respective forward and reverse primers. Sequences were assembled by Staden (Staden, 1996) to produce consensus sequences.

2.7. Reverse transcription quantitative PCR (RT-QPCR)

2.7.1. Primer design

Primers for RT-QPCR were designed for chicken and duck TENP (Table 2.1) and chicken, turkey, duck and zebra finch ovodefensin genes (Table 2.2). Primers were designed using the default settings of Primer3 (<http://frodo.wi.mit.edu/primer3/>) with a product length of 100-200 bp. Where possible primers were designed to span an intronic region to prevent amplification of genomic contamination, however in some cases the PCR primers used for whole sequence amplification were used for RT-QPCR for the ovodefensin genes (Table 2.2), however all RNA was treated with DNase before reverse transcription (see 2.5.2) and no genomic contamination was detected.

2.7.2. Standard curve production and experimental procedure

PCR was carried and single bands excised and purified from the agarose gel as outline in 2.6.2. Purified cDNA was diluted 1/500 the top standard which was detectable at around 8 cycles during RT-QPCR amplification and six ten-fold serial dilutions from this formed the standard curve.

Reverse transcribed samples (2.5.2) were diluted by a factor of 10 with MilliQ H₂O prior to use. RT-QPCR was carried out with 10 µl of the diluted cDNA and a primer

concentration of 20 mM according to Agilent Brilliant II SYBR® Green QPCR master mix (Stratagene, UK) instructions. The following conditions were used for RT-QPCR; 95°C for 2 min, then 40 cycles of 95°C for 15s, 60°C for 30s using an MX3000 (Stratagene, UK). Reactions containing no template were run as a control. Products were run on an agarose gel to confirm only products of the correct length with no primer-dimer were amplified as well as ensuring that there was only a single peak dissociation curve, correct amplification was also confirmed through sequencing of the PCR product. Lamin B-receptor (LBR) expression was shown previously to have only minimal variation between samples and was measured in the same way to normalise concentrations (McDerment et al., 2012).

2.7.3. RT-QPCR statistical analysis

One way or two way ANOVA and least significant difference to test between the means were used as appropriate for statistical analysis of log transformed data. (Genstat 13th edition, VSN International Ltd, Oxon, UK).

2.8. Rapid amplification of cDNA ends (RACE)

2.8.1. 5'RACE

5'RACE (Roche Diagnostics 2nd Generation, Mannheim, Germany) was carried out to determine the transcriptional start site of chicken *TENP*. Briefly, synthesis of first strand cDNA was carried out on magnum RNA using primer TENP SP1 (Table 2.1) and the mRNA template degraded. cDNA was purified using a High Pure PCR Product Purification kit (Roche Diagnostics, Mannheim, Germany) and polyA tailed at the 3' end. The tailed cDNA was amplified by PCR using the Oligo (dt)-anchor primer provided and a further nested primer TENP SP2 (Table 2.1). The product from this PCR was run using 3% agarose-gel electrophoresis and visualised using SYBR Safe DNA gel stain (Invitrogen, UK). PCR product was excised from the gel and cleaned from the agarose using a QIAEX II Gel Extraction Kit (Qiagen, UK) (see 2.6.2) and sequenced using primer SP2.

5'RACE and 3' RACE (see 2.8.2) were also carried out to determine the number of exons encoding *Gallus gallus OvoDA1 (gallin)*. The reaction was carried out as for

TENP using primers OvoDA1SP1 and OvoDA1SP2 (Table 2.2) and sequenced using primer OvoDA1SP2.

2.8.2. 3'RACE

3'RACE (Roche Diagnostics 2nd Generation, Mannheim, Germany) used the oligo(dT)-anchor primer to initiate cDNA synthesis at the poly(A)-tail of magnum RNA. Amplification using a PCR anchor primer and OvoDA1SP5 (Table 2.2) was then performed directly. PCR product was excised from the gel and cleaned from the agarose as before (2.6.2) and sequenced using primer OvoDA1SP5.

2.9. Sequencing and database submission

All sequencing was carried out by GATC biotech (Konstanz, Germany) and consensus sequences were submitted to EMBL.

Chicken *TENP* (EMBL accession no. HG007958)

Turkey *TENP* (EMBL accession no. HG425203)

Duck *TENP* (EMBL accession no. HG425202)

Gallus gallus OvoDB1 (EMBL accession no. LN717248)

Meleagris gallopavo OvoD1_2 (EMBL accession no. LN717249)

Meleagris gallopavo OvoDB1 (EMBL accession no. LN717250)

Taeniopygia guttata OvoDA1 (EMBL accession no. LN717251)

Taeniopygia guttata OvoDA1_2 (EMBL accession no. LN717252)

Taeniopygia guttata OvoDB1 (EMBL accession no. LN717253).

Putative ovodefensin sequences for budgerigar, medium ground finch, anole lizard, american alligator, collared flycatcher, painted turtle and chinese soft-shelled turtle were not submitted because they remain predicted but can be found in Chapter 4, Table 4.2.

2.10. Northern analysis

RNA was subject to electrophoresis on a 2% agarose gel under denaturing conditions (Pelle and Murphy, 1993) and SYBR safe DNA gel stain (Invitrogen, UK) was used for visualisation. The RNA was then transferred to an Amersham Hybond N+ nylon membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) after washing the gel in dH₂O for 30s; 50 mM NaOH, 10 mM NaCl for 45 min; 0.1 M Tris HCl pH 7.5 for 45 mins; 20xSSC for 60 mins and UV-crosslinked (Stratagene Stratalinker) at 120,000 microjoules/cm². A Riboprobe In-vitro Transcription Systems kit (Promega, Southampton, UK) was used to produce a single stranded RNA probe via the T3 RNA polymerase system and incorporated ³²P labelled UTP. Hybridisation of ³²P probe was detected using the Typhoon FLA7000 (GE Healthcare Life Sciences, Buckinghamshire, UK).

2.11. Production and titres of polyclonal antibodies

Production of antibodies was carried out by Dundee Cell Products Ltd, Dundee. Briefly, two rabbits per peptide were immunised four times at three week intervals by intramuscular injection of synthesised *Gallus gallus* OvoDB1 (R108 and R109) epitope (CNKKDEWSFHQ), *Taeniopygia guttata* OvoDB1 (R112 and R113) epitope (KGEREEHTED) or TENP (R106 and R107) epitope (AWMDDVLREGVHLPHLSH and DAELSLAASNVGLVRAA) emulsified in Freund's adjuvant. Serum was collected after each immunisation. Antiserum was purified via a two-step affinity purification using cognate peptides coupled to beads.

To measure the titres of antibody in the antisera, the synthesised epitopes were diluted with 50 mM Na₂CO₃ (pH 9.6) to a final concentration of 1 ng/μl (0.5 ng/μl of each epitope) and 50 μl of the solution was added to each well of a 96 well plate. The plate was covered and stored overnight at 4°C. This was aspirated and the wells incubated for 2 hours at room temperature with 200 μl tris-buffered saline (pH 7.5), 0.5% Tween 20 (TBST), 1% bovine serum albumin (BSA) to block unsaturated binding sites. Pre-immune (null) sera and antisera were serially diluted 1/1000 to 1/32000 with TBST, 1% BSA, pH 7.5. To each well, 10 μl of diluted null sera or

antisera were added and the plate incubated for 2 hours at room temperature. The plate was washed 5 times with TBST. Horse radish peroxidase (HRP) conjugated Anti-rabbit IgG (SAPU, Edinburgh, Scotland) diluted 1/2000 with TBST, 1% BSA, pH 7.5 100 µl was applied to each well and the plate incubated at room temperature for 2 hours. After five washings with TBST, peroxidase activity was detected by adding 100 µl detection solution (100 mM citric acid, 200 mM Na₂HPO₄, O-Phenylenediamine (OPD), H₂O₂). The reaction was stopped with 50 µl 2 M H₂SO₄ and absorbance (490 nm) measured spectrophotometrically.

2.12. Immunohistochemistry

All immunohistochemistry was carried out in collaboration with Maureen Bain and Lynn Stevenson at the College of Medical, Veterinary and Life Sciences (MVLS), University of Glasgow, Glasgow, Scotland, G61 1QGH, UK using the antibodies from 2.11. Chicken tissues (magnum, isthmus, shell gland and caecum) were harvested from five laying hens post mortem (Gong et al., 2010) or zebra finches (magnum, isthmus, shell gland and breast muscle) (see 2.4.5) and fixed in 10% buffered-neutral formalin (BNF) for 24 hours prior to being processed to paraffin wax using a 16 hour processing cycle in a Thermoshandon Excelsior tissue processor.

2.12.1. TENP

Wax embedded tissues were sectioned at 3 microns using a Thermoshandon Finesse microtome, lifted onto vecta slides and incubated at 60°C for 1 hour before they were de-waxed and taken down to water. Each section was then treated with Proteinase K for 20 minutes at room temperature (antigen retrieval) before loading onto a Dako Autostainer (Dako, Cambridgeshire, UK). A standard IHC protocol was then used; optimal staining was achieved at a 1:3000 dilution of the polyclonal anti-TENP antiserum (107_AWM_1.1) for 30 minutes. The sections were viewed using a Leica DM 4000 B microscope and images captured using a Leica DC480 camera with Qwin program for PC (Leica, London, UK).

2.12.2. *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1

Wax embedded tissues were sectioned at 3 microns using a ThermoShandon finesse microtome, lifted onto Vetabond slides and incubated at 60°C for 1 hour before they were de-waxed and taken down to water. Heat induced epitope retrieval (HIER) was carried out using a Menarini Access Retrieval Unit, in buffer (Sodium Citrate pH 6) for 1 minute 40 seconds at 125°C full pressure. Each section was then loaded onto a Dako Autostainer (Dako, Cambridgeshire, UK). A standard IHC protocol was then applied; optimal staining was achieved at a 1:500 dilution for the polyclonal zebra finch anti-OvoDB1 (113_KGE_2.1) and 1:1000 for the chicken anti-OvoDB1 (108 CNK-1.3). The sections were viewed using a Leica DM 4000 B microscope and images captured using a Leica DC480 camera with Qwin program for PC (Leica, London, UK).

2.13. Synthetic peptide production

Gallus gallus OvoDA1 (LVLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLPASWKWK), OvoDB1 (KRKGTCKGYCAPTCNKKDEWSFHQSCKKMYCCLPLKKGK) and *Anas platyrhynchos* OvoDA1 (QVRKYCPKVGYCSSLKSKADVWSLSSDCKFYCCLPPGWKGK) were commercially obtained from Almac Group (East Lothian, Scotland). The peptides were synthesized on a 0.2 mmol scale using an automated Applied Biosystems 433 peptide synthesizer and fluorenylmethoxy (Fmoc) solid phase peptide synthesis protocols. Each amino acid was coupled after activation with diisopropylcarbodiimide/Oxyma pure. Upon completion of the synthesis the peptide was cleaved from the resin and the side chain protecting groups removed using a cocktail of trifluoroacetic acid, ethanedithiol triisopropylsilane and H₂O. The peptide was folded in the presence of oxidised and reduced glutathione at pH 8 and the final product isolated using preparative HPLC and a gradient of H₂O, acetonitrile and 0.1% trifluoroacetic acid. Freeze dried peptides were reconstituted in 10% dimethyl sulfoxide (DMSO).

2.14. Recombinant peptide production

Recombinant *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1 were produced by Roal, Finland using a continuous fungal fermentation system. For production information sheets were given to Roal for each gene (Appendix 2) which contained cDNA and amino acid sequences for the mature peptide to be produced as well as detailed peptide information including molecular weight, charge, isoelectric point and inclusion body probability. Modified *Trichoderma* sp. with reduced endogenous protein production expressing a vector containing each ovodefensin was used as the production organism and the resulting supernatant was purified further using molecular weight filters of <3 kDa and >50 kDa before being spray dried to powder form for the final product. A *Trichoderma* system expressing an empty vector was treated in the same way to act as a control in the *in vitro* and *ex vivo* evaluation.

Due to the confidential nature of the system further details on production cannot be provided in this thesis.

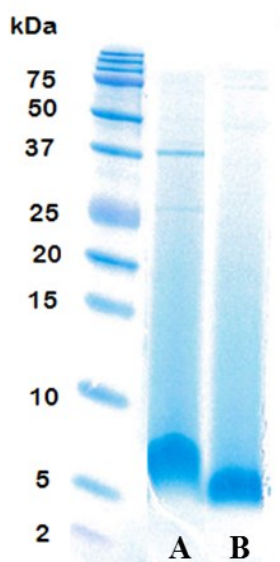


Figure 2.1: SDS-PAGE on Criterion 16.5% Tris-Tricine gel of recombinant peptide samples. *Taeniopygia guttata* OvoDB1 (A) and *Gallus gallus* OvoDA1 (B) were confirmed using N-terminal peptide sequencing.

2.15. Antimicrobial assay

The antimicrobial assay, adapted from methods described previously (Nagaoka et al., 2000, Townes et al., 2004, Gong et al., 2010) was used to determine the efficacy of synthetic *Gallus gallus* OvoDA1, OvoDB1 and *Anas platyrhynchos* OvoDB1. *E. coli* K-12 strain DH5 α , avian pathogenic *E. coli* (APEC) O78:H9 strain χ 7122, *Salmonella enterica* serovars Enteritidis (SE125109) and Typhimurium (ST4/74) and *Staphylococcus aureus* (8325-4) were cultured overnight at 37°C in Luria broth (LB) or tryptone soya broth (TSB) (*S. aureus*). Two hundred and fifty μ l of overnight culture was sub-cultured into 20 ml of LB or TSB and incubated at 37°C for 3 hours. After the second incubation 20 μ l of culture was diluted with 2 ml of phosphate buffered saline (PBS), pH 7.4. Ten μ l of *Gallus gallus* OvoDA1, OvoDB1 or *Anas platyrhynchos* OvoDB1 peptide or DMSO (control) or PBS was added to 50 μ l of diluted culture. After vortexing this was incubated at 37°C for 3 hours and then the suspensions were serially diluted to 1×10^{-4} with PBS, all dilutions were plated on LB or tryptone soya agar plates. Plates were incubated overnight at 37°C and the colonies were counted. Results are expressed as a reduction in colony forming units per ml (CFU/ml).

2.15.1. *Campylobacter jejuni*

Synthetic *Gallus gallus* OvoDA1 was also evaluated against a strain of *Campylobacter jejuni* 11168 known to possess genes (cj0423 and cj0424) which are thought to confer resistance to cationic antimicrobial peptides as well as a mutant of this strain (11168H) which lacks these genes (Unpublished data, supplied by Cosmin Chintoan, Roslin Institute). The same broth based assay as described previously (2.15) was used but MH broth was used in this assay and blood free campylobacter selective agar containing CCDA selective supplement was used for enumeration of *C. jejuni* colonies. Overnight cultures were grown in a microaerophilic cabinet at 400 rpm in pre-equilibrated media. Plates for enumeration were counted after a 2 day incubation period in microaerophilic conditions.

2.15.2. pH sensitivity

The antimicrobial assay as outlined above (2.15) was adapted to test the effect of pH on the efficacy of synthetic *Gallus gallus* OvoDA1 against *E. coli* DH5 α . The assay was carried out as before but using PBS at pH 6.4, 7.4 and 8.4 (Appendix 1).

pH sensitivity was carried out by Tian Chee Lu as part of a summer placement project which I designed and supervised.

2.15.3. Salt sensitivity

The antimicrobial assay (5.3.2) was adapted to test the effect of salt sensitivity on *Gallus gallus* OvoDA1 efficacy. PBS with NaCl concentrations of 50, 100 and 150 mM, pH 6.4 were used (Appendix 1).

Salt sensitivity was carried out by Tian Chee Lu as part of a summer placement project which I designed and supervised.

2.15.4. Bacterial metabolic state

Some traditional antimicrobials are only able to kill replicating bacteria. To determine if metabolic state has an effect on synthetic *Gallus gallus* OvoDA1 activity it was assessed against Avian Pathogenic *E. coli* in both a metabolically active and static state. Ampicillin was used as positive control in these experiments as it is known to interfere with cell wall synthesis and therefore requires bacterial cells to be metabolically active (Kong et al., 2010a). The standard assay produces bacteria in a metabolic state and was carried out as before (2.15) to produce metabolically active APEC. To produce static APEC the culture medium was centrifuged at 10,000 rpm for 5 minutes, the LB broth removed and the bacterial pellet resuspended in the same volume of PBS. This step was repeated three times to ensure all traces of LB nutrient were removed from the system before the bacteria was incubated in PBS with *Gallus gallus* OvoDA1.

2.16. Viral plaque assay

The antiviral activity of synthetic *Gallus gallus* OvoDA1, OvoDB1 and recombinant *Taeniopygia guttata* OvoDB1 was evaluated against a H1N1 Influenza A virus (A/WSN/33) (obtained by Gareth Hardisty, Roslin Institute) using a eukaryotic MDCK (Madin-Darby canine kidney) cell monolayer. A known antiviral peptide FluPep (Nicol et al., 2012) (courtesy of Seema Jasim, Roslin Institute) was used as a positive control. FluPep is a small peptide of 2.785 kDa which blocks the uptake of influenza virus into cells by interacting with haemagglutinin and preventing virus to cell binding.

Monolayers of MDCK cells were grown to confluence in six-well dishes and infected with 250 plaque forming units of influenza virus per well. Virus and cells were incubated in the presence or absence of peptide at 37°C with 5% CO₂, in a total volume of 400 µl. Peptide and virus were mixed immediately prior to addition to the cell monolayer; 10% DMSO was used as a negative control. After 1 hour unbound virus and/or peptide were removed and a 1% agarose in DMEM/*N*-acetylated trypsin was overlaid onto the cell layer. The cell monolayers were fixed with 10% neutral buffered formalin, stained with 0.1% toluidine blue and the plaques were counted after 3 days. Results were expressed as a reduction in plaque forming units when compared to virus alone.

2.17. Macrophage infection assay

The ability of *Gallus gallus* OvoDA1 at 1 and 10 µM to directly activate a chicken macrophage-like clonal cell line (HD11) was evaluated using *Salmonella*-gentamicin assay. One million HD11 cells were added per well in a six-welled dish and incubated at 37°C, 5% CO₂ overnight. Peptide or 10% DMSO was incubated with the cells for 2 hours and then removed. 10 µl of *Salmonella* (10⁶) was added to each well, swirled gently, centrifuged at 400 rpm for 6 minutes and incubated at 37°C, 5% CO₂ for 45 min to allow interactions to take place. After incubation 2 µl gentamicin (50 mg ml⁻¹) was added to give a final concentration of 100 µg ml⁻¹ and incubated for 90 min at 37°C, 5% CO₂. Cells were washed twice with Hanks solution (2-3 ml) to

remove gentamicin and 1 ml Hanks, 0.1% Triton X100 was added to lyse the cells. Lysates were serially diluted and plated on LB agar plates to determine number of intracellular bacteria; this represents net total number of *Salmonella* phagocytised by the macrophages as well as the intracellular *Salmonella* replication of those phagocytised. Results are expressed as a % reduction in *Salmonella* colony forming units compared to the negative control (10% DMSO).

2.18. *Ex vivo* gut model

This method was adapted for poultry from work published on pigs (Apajalahti et al., 2009). The *ex vivo* model and analysis of pH, gas and bacterial metabolites, which will be referred to as environmental analysis from this point on was carried out by Alimetries, Finland. I assisted with the experimental analysis of the microbial community and carried out the analysis shown in this thesis.

Conditions for the *ex vivo* intestinal simulation study were adjusted to mimic chicken ileal and caecal conditions as accurately as possible. For the simulations, recombinant *Gallus gallus* OvoDA1 was tested at 0.0047, 0.047 and 0.47 g/kg and *Taeniopygia guttata* OvoDB1 was tested at 0.0045, 0.045 and 0.45 g/kg of dry peptide product. These gave approximate final concentrations of 50, 100 and 200 μ M; the control supernatant was tested at 0.047 g/kg, a dose equivalent to 100 μ M of recombinant peptide. A negative control containing no additional supplement was included for comparison.

For preparation of the growth medium for the ileum simulation, digesta was collected from a section of the ileum ranging from the meckel's diverticulum to the ileal-caecal junction of thirty 4-week old broiler chickens. The digesta was mixed 1:1 with the ileal buffer (see appendix 1) and centrifuged at 18000 x g for 20 minutes to pellet the solids. The supernatant from this step was removed and mixed 11:1 with ileal buffer to create the ileal medium for all ileum simulations. All transfer steps were carried out in an anaerobic glove box and reagents were kept under anaerobic conditions. Ileum digesta inoculum (untreated from original collection) which had been kept anaerobically and used within 4 hours of collection was mixed with the

ileum medium at 0.1%. Immediately 10 ml aliquots of the ileum medium-inoculum were introduced anaerobically to fermentation vessels containing pre-weighed test compounds.

For preparation of the growth medium for the caecal simulation, digesta was collected from the caeca of thirty 4-week old broiler chickens. The digesta was mixed 1:1 with the caecal buffer (see appendix 1) and centrifuged at $18000 \times g$ for 20 minutes to pellet the solids. After centrifugation the supernatant from this step was mixed with ileal medium and caecal buffer in a ratio (vol/vol) of 3.5:1.5:5.0 to create the caecal medium for all caecal simulations. Again all transfer steps were carried out in an anaerobic glove box and reagents were kept under anaerobic conditions. Caecal digesta inoculum (untreated from original collection) which had been kept anaerobically and used within 4 hours of collection was mixed with the ileum medium at 10%. As before 10 ml aliquots of the caecal medium-inoculum were immediately introduced anaerobically to fermentation vessels containing pre-weighed test compounds.

Fermentation vessels were closed with thick butyl rubber stoppers, transferred to 37°C and continuously mixed in a gyratory shaker at 100 rpm. The simulations had five replicate vessels for each treatment, and the inoculation was done in a random order to avoid any potential systematic shifts. Incubation was continued for 10 hours prior to sampling of the vessels for various analyses.

At samplings, the total gas production was measured by puncturing the rubber stopper with a needle connected to an accurate 20 ml glass syringe with a sensitive ground plunger. The volume of gas released from the vessels was recorded. After opening the simulation vessel, the pH of the fermentation medium was measured with a calibrated pH meter. This measurement was done immediately after the gas measurement to avoid pH shifts caused by the escape of CO₂ from the medium. The medium was then sampled for the analysis of short chain fatty acids (SCFA) and bacterial density. The SCFA were analysed as free acids by gas chromatography, using pivalic acid as an internal standard, as described elsewhere in detail (Holben et

al., 2002). The acids measured were acetic, propionic, butyric, iso-butyric 2-methyl-butyric, valeric and lactic acid.

For bacterial quantification the bacteria in the samples were separated by differential centrifugation, bacterial cell walls disrupted, and the chromosomal DNA quantitatively purified using the Alimetrics in-house protocol. Total bacteria and specific bacterial species were quantified using q-PCR.

Treatments were compared using ANOVA to identify significant differences. To determine if the differences were peptide specific or a result of background fungal components the Least Significant Difference (LSD) test was used to compare the negative control, supernatant control and 100 μ M treatments.

2.19. *In vivo* feed trial

To test the hypothesis that the inclusion of ovodefensin peptide in poultry feed can improve growth performance of poultry through the modulation of the gut microbiome and environmental profile *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1 peptide produced using the recombinant fungal system outlined in 2.14 was administered to chickens in the feed. The negative control for this experiment was the absence of peptide from the feed.

Trial design was carried out jointly by Alimetrics, AB Vista and me; I carried out the feed formulation under the supervision of AB Vista. This experimental feed trial was carried out at Alimetrics, Finland and the sample collection was carried out according to my instruction. I carried out the analysis shown in this thesis.

2.19.1. Animals and housing

Newly-hatched male Ross 508 broiler chicks (HKScan, Finland) were randomly allocated to the feeding treatments. Birds were housed in 64 open pens (1.125 m² each) with wood shavings litter, 17 chicks per pen, 8 replicate pens and 1088 birds in total. The mean weight of the hatchlings in the pens was set between 38 g and 45 g. Chicks with compromised health were excluded from the trial.

Feed and water were available *ad libitum* at all times during the trial. Water was provided using nipple watering lines and feed was provided in half bell shaped feeders attached to the side of the pen.

On day 1, the lights were on for 24 hours. From day 2, the dark period increased an hour per day until on day 7 there was 18 hours of light and 6 hours of dark per day. This 18:6, light:dark cycle was continued until the end of the trial. On day 1, the temperature was set to 29°C, and heat bulbs were used to give additional warmth. The light bulbs were kept on for the first seven days, after the first week the temperature was dropped by 0.5°C by day.

All welfare standards complied with The Finnish Act on Animal Experimentation.

2.19.2. Feed formulation

The diet was a wheat-soya based commercial-type mash feed for broiler chicks. A starter formula was used during the 3-week trial. The main feed ingredients were analysed for basic nutrients and amino acid profiles for the final formulation which can be found in Table 2.3.

The test substances were carefully mixed by hand into 5 kg premixes with wheat. Each premix batch will replace 5 kg of wheat from the final formula. The feeds were manufactured at Agri-Food Research, Finland. Three grab samples of 200 g each per diet from start, mid-point and the end of the diet manufacture run were collected, mixed and 300 g sent for analysis of moisture, crude protein, crude fibre, oil, ash, calcium and phosphorus to an accredited laboratory.

2.19.3. Sample collection

The chicks were weighed on days 1, 11 and 21. Correspondingly, feed intake per pen and the feed conversion ratio (FCR) was measured for the following periods: days 1-11, days 11-21 and days 1-21. The birds were monitored on a daily basis and any dead birds or birds euthanized because of health problems were weighed. Daily mortality was recorded and FCR was calculated both corrected and uncorrected for mortality.

On day 11, two birds per pen were weighed and euthanized by cervical dislocation. The abdominal cavity was opened, and the entire ileum and the paired caeca removed, packed in individual plastic bags, frozen immediately and stored at -20°C until analysis. On the day 21, a similar set of samples were collected, and all the remaining birds were weighed and euthanized.

2.19.4. Metabolite and intestinal environment analysis

The SCFAs were analysed as free acids by gas chromatography, using pivalic acid as an internal standard, as described elsewhere in detail (Holben et al., 2002). Total SCFAs, acetic, propionic, butyric, iso-butyric, 2Me-butyric, valeric, isovaleric and lactic acid were measured from the highest dose of each peptide and treatments were compared using a Kruskal-Wallis test as the data could not be normalised using typical transformations. Where significance was observed a Mann-Whitney ranked test was used to compare peptide treatments to the control.

2.19.5. Microbiome analysis

For DNA extraction the bacteria in the samples were separated by differential centrifugation, bacterial cell walls disrupted, and the chromosomal DNA quantitatively purified using the Alimetrics in-house protocol, which is optimised and validated for the bacterial DNA extraction from chicken digesta samples. In the initial washing step of the samples the major target is to remove feed particles and complex polysaccharides, which disturb the subsequent DNA purification process and the downstream PCR applications.

Bacterial numbers were measured using quantitative real time PCR. In this study, all results are expressed as a number of ribosomal RNA (rRNA) gene copies per 1 g of digesta. In order to estimate the number of chromosomes/genomes in the analyses the results were divided by the average rRNA copy number of the target organisms. For example for *E. coli* the copy number is 7, while *Eimeria* genome encodes roughly 100-200 copies rRNA depending on the species. Measurements were made for the highest dose of each peptide treatment and the negative control and significance was tested using Kruskal-Wallis as the data could not be normalised using typical

transformations. Where significance was observed a Mann-Whitney ranked test was used to compare peptide treatments to the control.

A	Ingredient	Inclusion (g/kg)
	Wheat - Feed	572.7
	Rapeseed Solvent Extract	60.0
	Soybean meal 48	286.6
	Sunflower oil	44.2
	Salt	3.7
	DL Methionine	2.6
	Lysine HCl	2.4
	Threonine	0.5
	Limestone	7.2
	Dicalcium Phos	15.3
	Vitamin premix	4.9

C	Nutrient	Starter (g/kg)
	Crude protein	223.2
	Calcium	9.0
	Phos	7.6
	Avail Phos	4.5
	Fat	5.9
	Fibre	30.9
	Met	5.9
	Cys	4.0
	Me+Cys	9.9
	Lys	13.4
	His	5.6
	Tryp	2.8
	Thr	8.7
	Arg	14.3
	Iso	9.1
	Leu	16.3
	Phe	10.3
	Tyr	7.4
	Val	10.1
Gly	9.3	
Ser	10.4	
Gly+ser	19.8	
Phe+Tyr	17.7	
Phytate P	2.1	
Na	1.8	
Cl	3.8	
K	9.1	
Linoleic acid	28.1	
DUA	334.1	
Sulphur	2.8	
Magnesium	1.8	
Choline	1.64	
Poult ME MJ/kg	12.55	
Poult NE Kcal/kg	2,023	
Poult ME kcal/kg	3,000	

B	Detailed composition of the vitamin premix	
	Calcium	331.33 g/kg
	Vitamin A	1800 mg/kg
	Vitamin D3	56.25 mg/kg
	Vitamin E	30,000 mg/kg
	E-tokoferol	27,270 mg/kg
	Vitamin K3	1,505 mg/kg
	Vitamin B1	1257.3 mg/kg
	Vitamin B2	3,000 mg/kg
	Vitamin B6	2009.7 mg/kg
	Vitamin B12	12.5 mg/kg
	Biotin	75 mg/kg
	Folic acid	504 mg/kg
	Niacin	20,072 mg/kg
	Pantothenic acid	7506.8 mg/kg

Table 2.3: Detailed composition of feed ingredients and analysis for *in vivo* feed trial (2.19). Inclusion of raw ingredients are shown in A; a detailed composition of the vitamin premix is shown in B and a breakdown of the overall nutrient composition of the starter diet is shown in C.

Table 2.3: Detailed composition of feed ingredients and analysis for *in vivo* feed trial (2.19). Inclusion of raw ingredients are shown in A; a detailed composition of the vitamin premix is shown in B and a breakdown of the overall nutrient composition of the starter diet is shown in C.

Chapter 3

Comparative biology and expression of TENP

3.1. Introduction

Poultry are crucial to global food security and until a recent ban under EU Directives in-feed antibiotics were used to promote energy retention from the diet and prevent microbial infections (Bedford, 2000). Antibiotic use has driven the evolution of transmissible antibiotic resistance that has reduced the effectiveness of human and veterinary medicines (Hancock, 1997). Despite restrictions, therapeutic antibiotic use remains possible but a pressing need exists to maintain the efficiency of animal production by finding alternatives. One of the critical needs in realising the potential of alternative antimicrobial strategies was identified as the characterisation of a wide range of molecules and their properties (Hancock and Sahl, 2006). Antimicrobial peptides have been suggested as an alternative due to their potential for rapid, broad spectrum bactericidal activity and low predisposition for resistance development (Hancock, 1997). The large abundance of natural antimicrobials make the egg an interesting reservoir of potential candidates particularly as the clinical application for this study relates to the food industry. Naturally derived egg products, as already widely consumed by humans, would offer a level of acceptability and therefore form the basis of the peptide portfolio for this study. One such potential candidate is the egg white protein ‘transiently expressed in neural precursors’ (TENP), however, as little is known about TENP or its function in the adult hen this was first characterised to determine if it is a suitable candidate.

The *TENP* gene was first identified in the brain and retina of developing neural tissues of chickens using reverse transcription polymerase chain reaction (RT-PCR), and was proposed to function in an early neurological event occurring in post-mitotic cells before they enter the stage of overt differentiation (Yan and Wang, 1998). The 47kDa protein has since been identified during proteomic studies of chicken egg white (Guerin-Dubiard et al., 2006, Mann, 2007, D'Ambrosio et al., 2008), vitelline membrane (Mann, 2008), shell (Mann et al., 2006) and yolk (Mann and Mann, 2008, Farinazzo et al., 2009). Although TENP expression in the embryo has been documented little is known about where, when and under what conditions it is expressed in the adult. *TENP* expression in the oviduct and caecum of the adult hen

had previously been reported by Chiang et al in 2011, yet both this and the embryonic work were qualitative studies which leaves unanswered questions regarding the predominant areas of expression in the adult hen and how this compares to levels in the embryo.

Similarity searches have shown homology of TENP with the bactericidal/permeability-increasing (*BPI*)-like family of innate immune genes (Yan and Wang, 1998), which are found in adjacent chromosomal regions to *TENP* on chromosome 20 (Chiang et al., 2011). Computer analysis of the primary sequence of *TENP* predicted three putative transmembrane helices suggesting it may be a membrane protein (Yan and Wang, 1998). This was supported by the immune detection of TENP in cellular membrane fractions after expression in chicken embryonic fibroblast cells (Yan and Wang, 1998). However, it has been suggested that TENP, as previously proposed for BPI, may be membrane associated rather than an integral membrane protein (Beamer et al., 1998). It has been proposed that *TENP* is a divergent ortholog of human *LPLUNC2* (Chiang et al., 2011). The PLUNC (palate, lung and nasal epithelial clone) protein family are structural homologues of BPI proteins (Chiang et al., 2011) and are divided into two groups; short (SPLUNC) and long (LPLUNC) proteins. SPLUNCs contain a region structurally homologous to the BPI N-terminal domain whereas LPLUNCs contain domains similar to both the BPI domains (Bingle and Craven, 2002). The N-terminal domain of BPI is responsible for lipopolysaccharide (LPS) and bacterial binding as well as endotoxin neutralisation and antibacterial cytotoxicity whereas the C-terminal domain is associated with opsonic effects thus enhancing phagocytosis (Schultz et al., 2001). The TENP molecule detected in the embryo has two distinct regions which demonstrated are analogous to the BPI1 (N-terminal) and BPI2 (C-terminal) domains of the BPI protein. However, there was little evidence from EST data (<http://genome.ucsc.edu/>) to support a full length *TENP* transcript in adult birds which would allow both the BPI-like domains present to be expressed in one molecule; yet the proteomic evidence supported both domains being present in egg white (Karlheinz Mann, Germany, personal communication, November 11 2010).

As each domain of the molecule is analogous to a BPI domain and therefore potentially associated with differing functions it was of interest to know how the TENP transcript is expressed in the adult bird.

It has been proposed that the function of TENP is associated with the innate defence of eggs against pathogens (Guerin-Dubiard et al., 2006, Mann, 2007, D'Ambrosio et al., 2008) however its proposed function had yet to be experimentally proven and is largely based on its relationship to the BPI-like family. A chicken member of the BPI/PLUNC super-family, is found in the egg shell and has previously been shown to possess modest antibacterial activity against a range of gram positive and gram negative bacteria (Gautron et al., 2011). It is also documented that this protein is able to bind *E.coli* LPS *in vitro* (Gautron et al., 2011) and its expression in the infundibulum is up-regulated after systemic administration of LPS (Bedrani et al., 2013) strongly supporting a role in egg antimicrobial defence. Although mammalian members of the LBP family have been documented to interact with LPS this has been demonstrated to be both pro and anti-inflammatory and the family's role in host defence against bacteria is still to be fully elucidated (Bingle and Craven, 2004). However, due to the large number of known antimicrobial proteins, including Ovocalyxin 36 (Gautron et al., 2011), already identified in the egg, and the known role of TENP in the emu egg (Maehashi et al., 2014), an antimicrobial role of TENP in the chicken would seem plausible.

3.2. Objectives

To test the hypothesis that TENP is predominately expressed in the magnum as one transcript encoding for both BPI domains in the adult hen and is under the control of gonadal steroids as expected for an egg specific protein. This study also aimed to identify TENP homologs across divergent bird species and to determine the suitability of TENP as a antimicrobial feed additive.

3.3. Methods

3.3.1. Bioinformatic analysis

The TENP protein sequence (NCBI accession no. AF029841) was used to perform a BLAT search (Kent, 2002) of the May 2006 release of the chicken EST database using the default parameters of the UCSC genome browser (<http://genome.ucsc.edu/>) to identify ESTs and sequences related to the locus. The Staden package (Staden, 1996) was used to build a consensus sequence using the ESTs available. This allowed a gap present in the genome build to be bridged and allowed a comparison to be made between the genomic, EST and published *TENP* mRNA sequences in order to address differences in sequence identity. Primer3 (<http://frodo.wi.mit.edu/primer3/>) was used to design primers (Chapter 2, Table 2.1) for re-sequencing across the length of the consensus sequence including a putative alternative transcriptional start site identified in the consensus sequence. Signal P (Bendtsen et al., 2004) was used to assess the likelihood of a signal peptide produced by each translational start site. Exon contributions were estimated from genomic DNA using BDGP NNSPLICE version 0.9 (Reese et al., 1997).

The duck genome database (*Anas platyrhynchos* – version 1) (<http://pre.ensembl.org>) and turkey genome database (*Meleagris gallopavo* – assembly UMD2) (<http://www.ensembl.org>) were searched using TBLASTN for potential homologues using the 629 amino acid mature TENP protein. Primers were designed using Primer3 (<http://frodo.wi.mit.edu/primer3/>) to amplify both the potential turkey and duck *TENP* sequences from cDNA (Chapter 2, Table 2.1).

3.3.2. Animals and tissue collection

To determine the location of *TENP* expression in the adult bird, tissues were taken from sexually mature White Leghorn LSL hens (*Gallus gallus*) (Lohmann), sexually mature Pekin ducks (*Anas platyrhynchos*) (Cherry Valley) and sexually mature turkeys (*Meleagris gallopavo*) (Kelly Bronze). Details of tissues taken can be found in section 2.4.

For quantitative comparison with embryonic expression brain, retina and heart tissue was taken from embryonic day 10 (E10) (n=6) and day 16 White Leghorn chickens (E16) (n=6). E10 and E16 correspond with Hamburger–Hamilton (HH) embryonic developmental stages HH36 and HH42 respectively.

In order to assess the effect of oviposition on TENP expression magnum tissue was obtained from sexually mature White Leghorn hens with an ovum at various positions in the oviduct, see section 2.4.7 for details.

TENP expression was measured in hens that were in lay (n=11) and those where the oviduct had regressed due to incubation behaviour (n=11) to establish the effect of oviduct development, (section 2.4.8).

The induction of *TENP* with steroid hormones was adapted from a method described previously by Kunas, *et al.* Details can be found in section 2.4.9.

3.3.3. RNA preparation

RNA was extracted from tissues and processed as per the Ultraspec protocol (section 2.5.1).

3.3.4. Transcript determination

3.3.4.1. PCR and sequencing

1 µg samples of chicken, duck and turkey magnum RNA were reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, UK) according to the manufacturer's protocol (2.5.2). Primers TENPExon1F1 and TENPEnd1 (chicken), D.TENPF3, D.TENP R3, D.TENP F8 and D.TENP R10 (duck) and T.TENPF1 and T.TENPR1 (turkey) (Chapter 2, Table 2.1) were designed to ensure complete coverage of the *TENP* sequence (see 3.3.1). PCR amplification was performed using standard conditions (section 2.6.2). Amplified PCR fragments were sequenced with the forward and reverse primers. Sequences were assembled by Staden (Staden, 1996) to produce consensus sequences.

A phylogram was constructed using Mega5 to infer the evolutionary history of the *TENP* homologues. Included in this analysis was human *LPLUNC2* (NCBI reference sequence NM_025227) and chicken *Ovocalyxin 36* a *BPI* like gene (NCBI reference sequence NM_001030861) as outliers.

3.3.4.2. Northern analysis

Magnum RNA (2 µg) from in-lay, layer (n=3) and broiler (n=3) lines and broiler liver RNA (12 µg) were electrophoresised on a 2% agarose gel under denaturing conditions (Pelle and Murphy, 1993), SYBR safe DNA gel stain (Invitrogen, UK) was used for visualisation. The RNA was then transferred to an Amersham Hybond N+ nylon membrane for northern analysis (section 2.10). Template DNA consisted of a *Hind*III linearised pBluescript plasmid (p347_TENP) containing the cloned *BPI2* (EMBL HG007958 position 1196-1569) domain present in both potential TENP transcripts. Hybridisation of ³²P probe was detected using the Typhoon FLA7000 (GE Healthcare Life Sciences, Buckinghamshire, UK).

3.3.4.3. 5'RACE

5'RACE (Roche Diagnostics 2nd Generation, Mannheim, Germany) was carried out using primers SP1 and SP2 to determine the transcriptional start site of *TENP* (section 2.8.1). PCR product was sequenced using primer SP2 (Chapter 2, Table 2.1).

3.3.5. Reverse transcription quantitative polymerase chain reaction (RT-QPCR)

1 µg of cDNA was prepared (section 2.5.2) and used for RT-QPCR analysis as detailed in section 2.7.2. Primer3 (<http://frodo.wi.mit.edu/primer3/>) was used to design primers TENP RTF and TENP RTR for amplification of chicken TENP; D.TENP F8 and D.TENP R10 for duck TENP (Chapter 2, Table 2.1). Reactions containing no template were used as a control. Lamin B-receptor (LBR) expression was measured in the same way to normalise concentrations as used previously (McDerment et al., 2012). One way or two way ANOVA and least significant

difference to test between the means were used as appropriate for statistical analysis of log transformed data. (Genstat 13th edition, VSN International Ltd, Oxon, UK).

3.3.6. Production and titres of polyclonal anti-TENP antibodies

Production of antibodies was carried out by Dundee Cell Products Ltd, Dundee (section 2.11); titres of anti-TENP in the antisera were measured (section 2.11) for use in immunohistochemistry.

3.3.7. Immunohistochemistry (IHC)

Wax embedded tissues were prepared (section 2.12), a standard IHC protocol was then used; optimal staining was achieved at a 1:3000 dilution of the polyclonal anti-TENP antiserum (107_AWM_1.1) for 30 minutes.

All immunohistochemistry was carried out in collaboration with Maureen Bain and Lynn Stevenson at the College of Medical, Veterinary and Life Sciences (MVLS), University of Glasgow, Glasgow, Scotland, G61 1QGH, UK.

3.3.8. Sequencing and database submission

All sequencing was carried out by GATC biotech (Konstanz, Germany) and consensus sequences were submitted to EMBL, chicken *TENP* (EMBL accession HG007958), turkey *TENP* (EMBL accession HG425203) and duck *TENP* (EMBL accession HG425202).

3.4. Results

3.4.1. Bioinformatic analysis and transcript confirmation

A BLAT search of the May 2006 chicken genome (<http://genome.ucsc.edu/>) using the published TENP protein sequence (NCBI reference sequence. NM_205026) returned a result indicating a 98.2% identity. However there was a gap in the genomic data between 10248912-10249705 bp on chromosome 20. Searching the EST and cDNA database produced the following sequence accession numbers; Genbank accession no. DT657251, BM439389, DT655485, DT655483, DT654774,

DT657764, BU357647, BX265690, BX265691, BU210629, BU266397 and AF029841. All sequences were derived from hen reproductive tract except AF029841 which was from the original *TENP* publication (Yan and Wang, 1998) that used embryos. These were aligned using Staden (Staden, 1996) and the consensus sequence produced was used to close the gap in the genomic sequence. The EST and genomic data either side of the gap were identical; the published sequence for *TENP* was 99.1% identical to the sequence generated by the Staden alignment. When the *TENP* PCR products generated in this study were sequenced they were identical to the available genomic and EST sequences. However they differed from the current RefSeq (NM_205026) by an insertion of G between positions 190-191 (NM_205026.1:c.190_191insG) and a deletion of G at position 251 (NM_205026.1:c.251delG) resulting in a partial frame shift, altering part of the protein sequence (Figure 3.1). Analysis of the sequence from this study (EMBL HG007958) identified a potential alternative translational start site (Figure 3.1). The published translational start site (Yan and Wang, 1998) at genome position Chr20:10,647,277 (reverse strand) of the November 2011 build with the signal peptide prediction MGALLALLDPVQPTRA gives a signal peptide probability of 0.661 and a max cleavage site probability of 0.651 whilst the new putative translational start site at position Chr20:10,647,544 (reverse strand) in the November 2011 build identified in this study results in a signal peptide prediction of MGTANRKGSAPVLLCTMGALLALLDPVQP that gives a stronger prediction for the signal peptide probability (0.996) and a maximum cleavage site probability of 0.534. 5'RACE using magnum RNA supported the presence of the transcript postulated in this thesis, which in turn supported the new translational start site (Figure 3.1). No evidence was found for a sequence which would support the previously published translational start site, however this may be expressed below the detection level of the methods used (Figure 3.1). The same method was used to detect the transcriptional start site used in embryonic brain tissue (not shown) however no transcript was detected.

It was noted that the EST data, although supporting the presence of each BPI-like domain in the reproductive tract, offered little evidence for their expression in one full length *TENP* transcript, rather supporting that the protein may be expressed as two separate molecules each encoding one of the BPI domains (Figure 3.2). PCR using magnum cDNA with primers TENP Exon1F1 and TENPEnd1 designed to amplify the whole of the *TENP* protein coding region from chr20:10642981-10647548 resulted in two strong bands. Sequencing of these PCR products confirmed that one of the transcripts encoded for a full length transcript including the new predicted translational start site, the second smaller transcript would if translated encode only the BPI2 region of the TENP protein (Figure 3.2). In order to confirm the expression of these *TENP* transcripts in magnum tissue northern analysis using an RNA probe corresponding to the BPI2 domain, present in both forms detected by PCR, was carried out. The results proved the presence of only the full length transcript in both layer and broiler type birds (Figure 3.1) but showed no indication of a smaller transcript despite the PCR evidence. In general the intensity of the signal from the layer line was lower and more variable than that of the broiler (Figure 3.1). In agreement with previous work (Chiang et al., 2011) it was predicted that chicken *TENP* has 16 exons (Table 3.1).

Potential *TENP* homologues were identified in both duck and turkey genomes and their expression was confirmed using PCR and sequencing. The phylogenetic analysis (Figure 3.4) suggests that *TENP* molecules from avian species are more similar to each other than either *Ovocalyxin 36* or *LPLUNC2*.

3.4.2. Tissue expression

TENP expression was detected in embryonic brain and retinal tissues (Figure 3.5) and decreased as development progressed from E10 to E16. In contrast measurement of *TENP* expression in adults indicated that expression was restricted to the oviduct of adult hens and almost exclusively the magnum with no detectable expression in either the brain or retina suggesting *TENP* plays a different role in the adult bird (Figure 3.6). Expression was also restricted to the magnum of the oviduct in the

Exon No.	Exon Contribution
1	1-53
2	54-170
3	171-270
4	271-375
5	376-510
6	511-556
7	557-617
8	618-709
9	710-885
10	886-928
11	929-1093
12	1094-1161
13	1162-1207
14	1208-1271
15	1272-1348
16	1349-1576

Table 3.1 – Exon Contributions. Exons are shown as nucleotide contributions in relation to the *TENP* sequence produced in this study (EMBL HG007958).

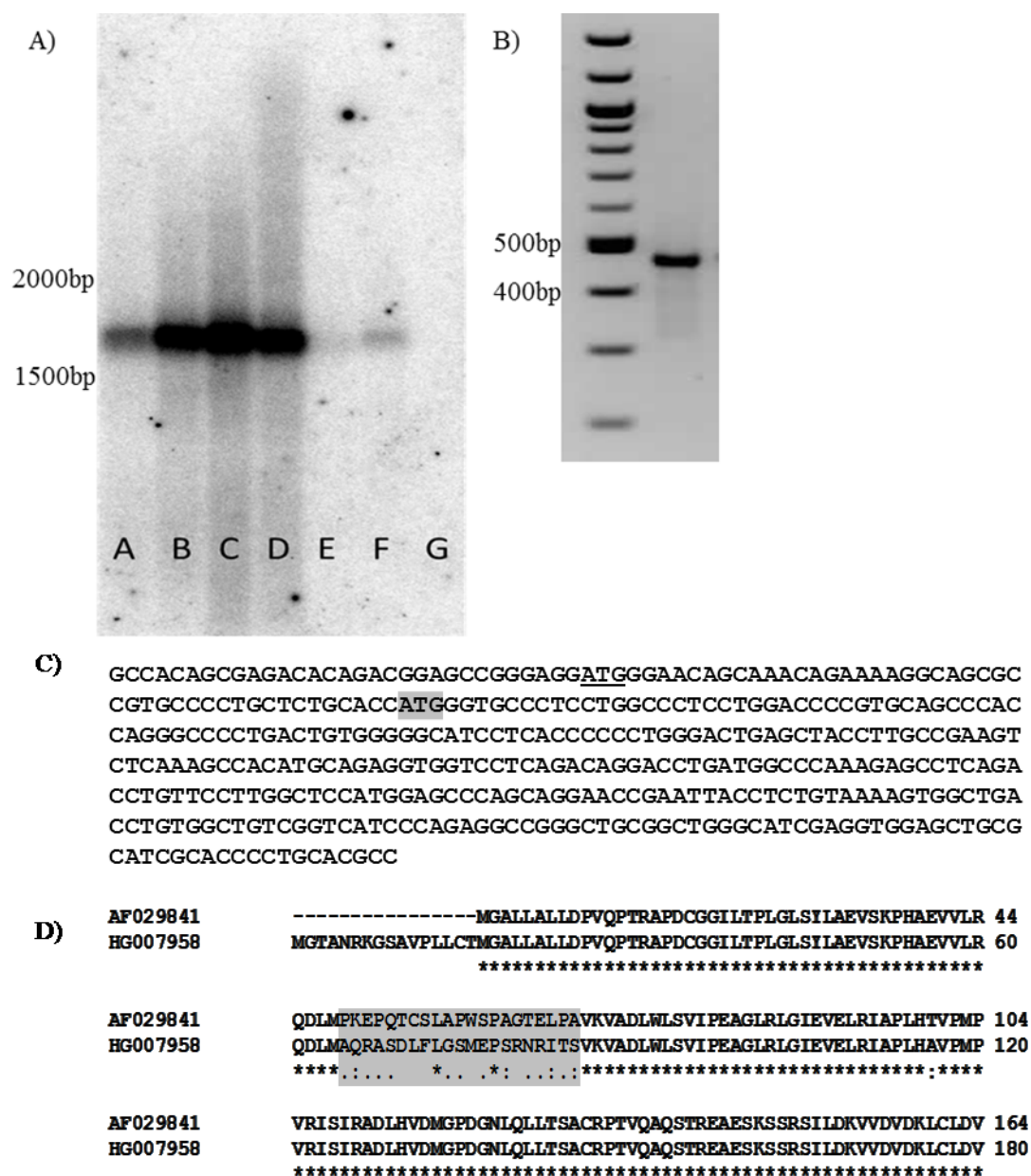


Figure 3.1 – Northern analysis and 5'RACE. A) Northern analysis of p347_TENP. Lanes A-C contain 2 µg broiler magnum RNA, lanes D-F contain 2 µg layer magnum RNA, 12 µg liver RNA (G) is used as a negative control. B) 5'RACE using primer SP2 resulting in one band. C) cDNA sequencing of the 5'RACE product from B confirmed the presence of an alternative translational start site (underlined), the published start site is highlighted (■). D) The first 180aa of the new putative protein (HG007958) translated from the alternative start site in C is shown in a clustal alignment with the published TENP sequence (AF029841); the location of the frameshift is highlighted (■). The proposed new protein has 455 amino acids.

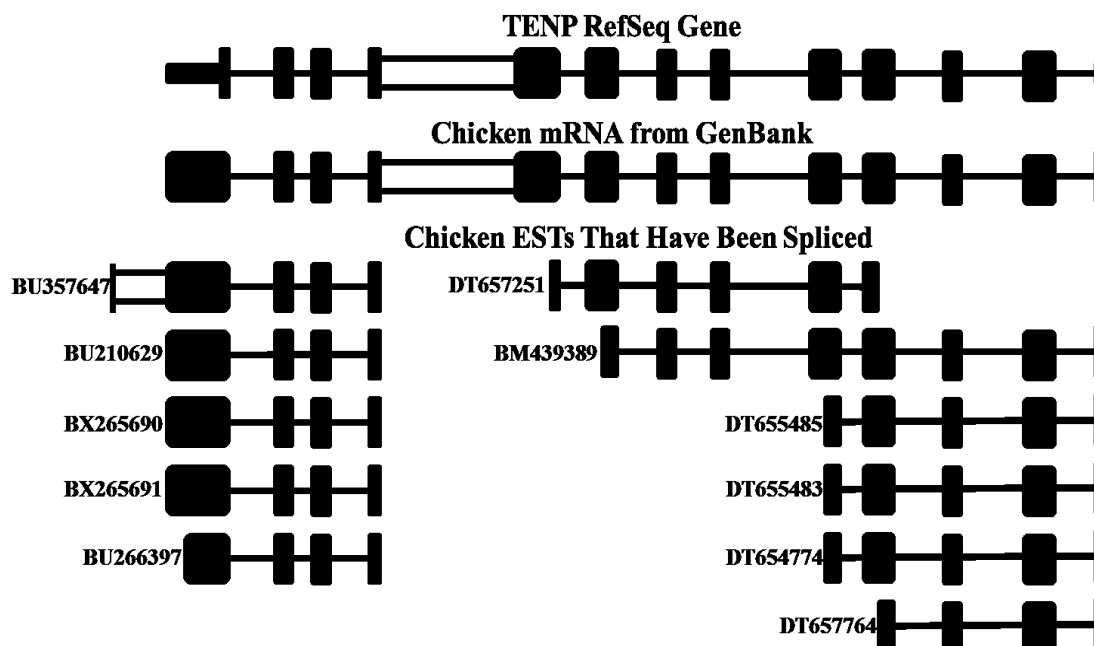


Figure 3.2 – Schematic representation of chicken ESTs using the EST data displayed by the UCSC Genome Browser website. ESTs were all isolated from the reproductive tract of adult hens and provided no evidence for a transcript where both BPI-like domains are expressed in one molecule.

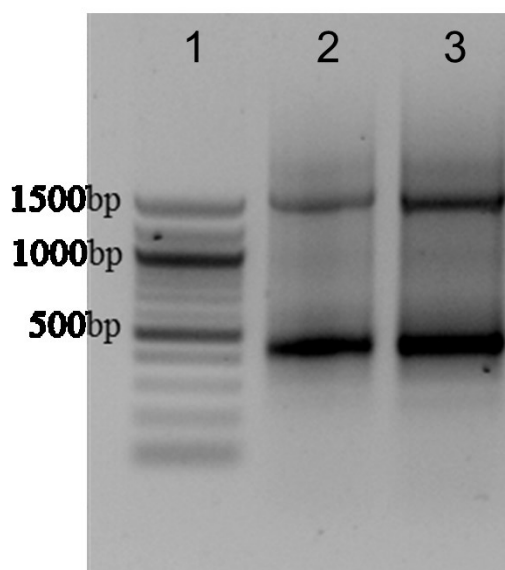


Figure 3.3 – *TENP* PCR – Amplification of *TENP* from magnum cDNA using primers TENP Exon1F1 and TENP End 1 in the first and last exon of *TENP*. Lane 1, 100 bp molecular weight marker; lane 2 and 3 magnum cDNA. Results indicate two *TENP* transcripts with sizes corresponding to a transcript encoding the full *TENP* protein and also that of just one BPI domain.

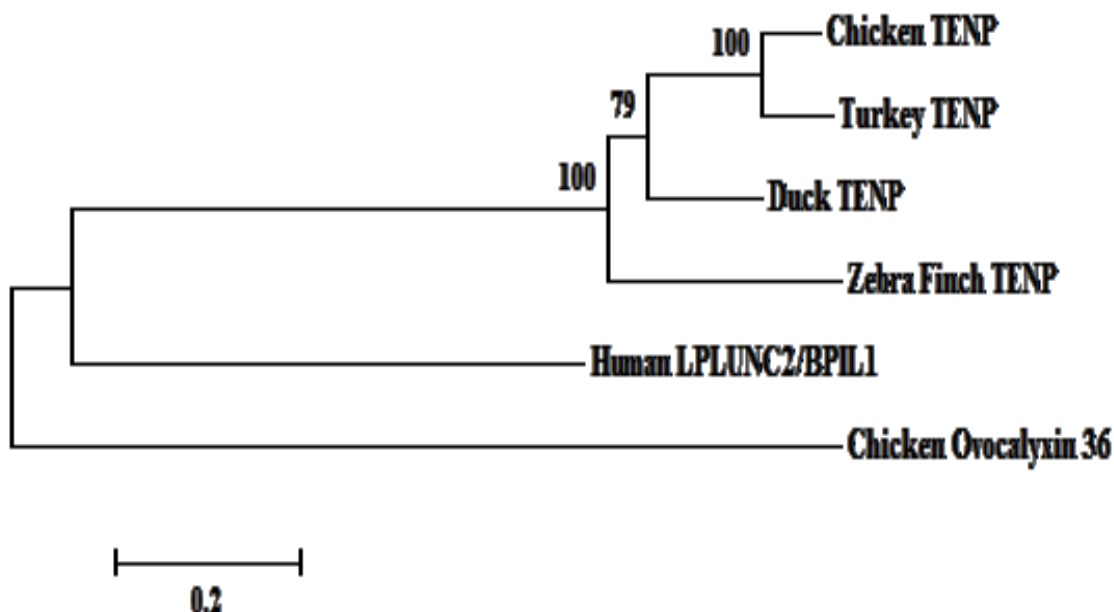


Figure 3.4 - Evolutionary relationships of avian *TENP* homologues, *LPLUNC2* and *Ovocalyxin 36*. A phylogram indicating the evolutionary history of *TENP* was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) from an alignment of the mature proteins. Included for comparison were human *LPLUNC2* and chicken *Ovocalyxin 36*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The branch lengths are proportional to the evolutionary distances which were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). The units of the branch lengths are the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The common name of the species is included with the protein names.

duck tissues sampled (Figure 3.6). *TENP* expression was measured in the magnum of the oviduct when the egg was either in the magnum, the shell gland or during a pause day. Eggs in the shell gland were classified as either early, mid or late depending on the level of calcification as determined by electron microscopy. The position of the egg in the oviduct or the occurrence of a pause day, when the hen did not ovulate and so no egg was present, did not significantly alter the level of *TENP* expression in the magnum (Figure 3.7) (ANOVA, $P=0.083$) although showed a clear trend. However if the data was categorised into when an egg was in the magnum or had just recently left it (early) versus the later stages of calcification (mid/late) then the difference is approaching significance (ANOVA, $P=0.051$). The level of *TENP* expression in both the magnum and the shell gland was higher ($P<0.001$) in birds in-lay than in adult hens whose oviduct had regressed due to incubation behaviour (Figure 3.8) which suggests that the level of *TENP* expression is affected by the reduction in gonadotrophins which in turn causes reduction in steroid secretion from the ovary. Oestrogen is well characterised as a key regulator in the development and function of the oviduct and is therefore unsurprisingly linked to regulation of the expression of many egg specific genes. When oestrogen and progesterone were administered to juvenile hens *TENP* expression (Figure 3.9) was higher in birds treated with the two steroids ($P<0.001$) and where priming with an oestrogenic compound had been performed overall expression increased ($P<0.001$).

3.4.3. Immunohistochemistry

The anti-*TENP* antiserum (107_AWM_1.1) produced positive staining in the tubular gland cells of the magnum (Figure 3.10 A). The ciliated and non-ciliated cells lining the magnum region of the oviduct did not react to the primary antibody. No staining was observed in the isthmus, shell gland or caecum (Figure 3.10 C-E).

3.5. Discussion

The conservation of *TENP* among avian species, and the results outlined in this study strongly suggest a major egg specific role for *TENP* protein in the adult hen. Although local tissue activity, for example antimicrobial protection of the oviduct

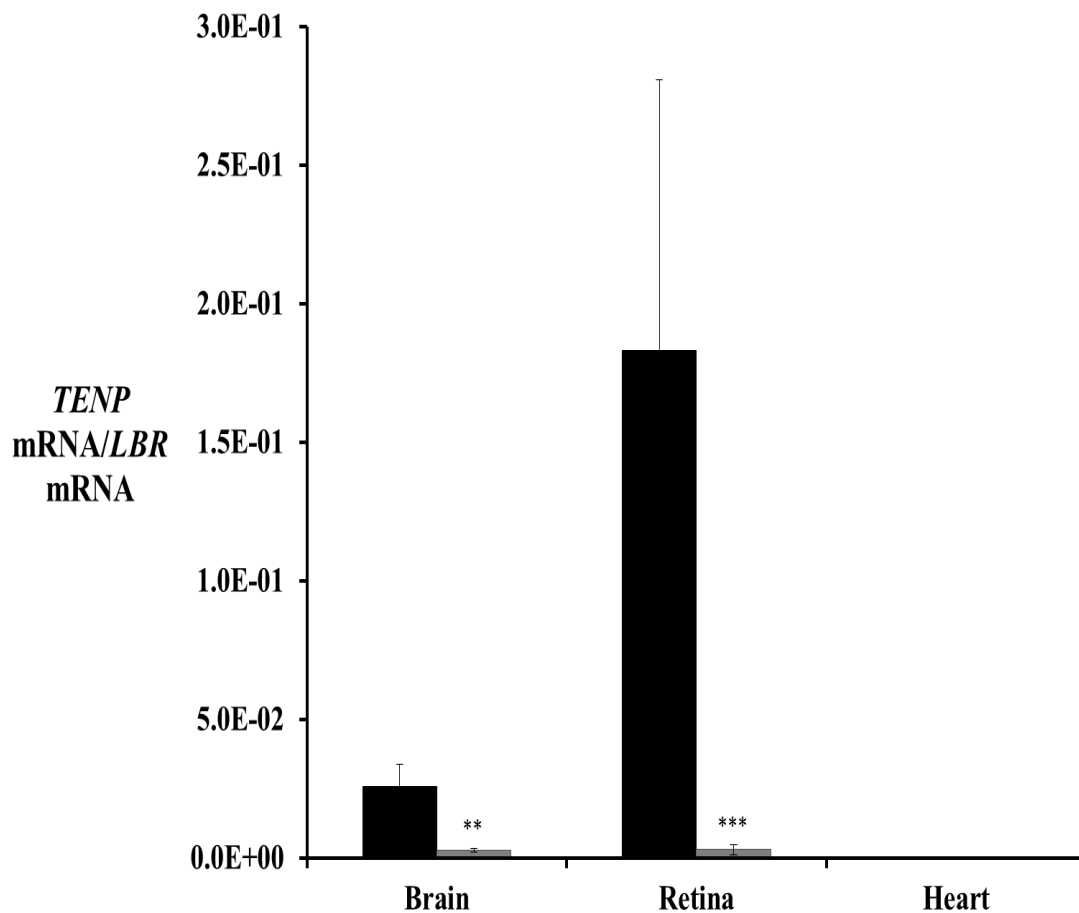


Figure 3.5 – Expression of *TENP* mRNA in embryonic day 10 (■) and 16 (■) chicken brain, retina and heart tissues measured by RT-QPCR (n=6, mean \pm sem). *TENP* expression was corrected for *LBR* expression to normalise for any differences between tissues in processing. ANOVA indicated that embryonic stage was significant at $P < 0.001$ and tissue was significant at $P < 0.001$. *TENP* expression clearly diminished with development. Heart was included as a negative control, as expected no expression was detected. Significance between embryonic stage within tissue is indicated $P < 0.001$ (*) and $P < 0.01$ (**).**

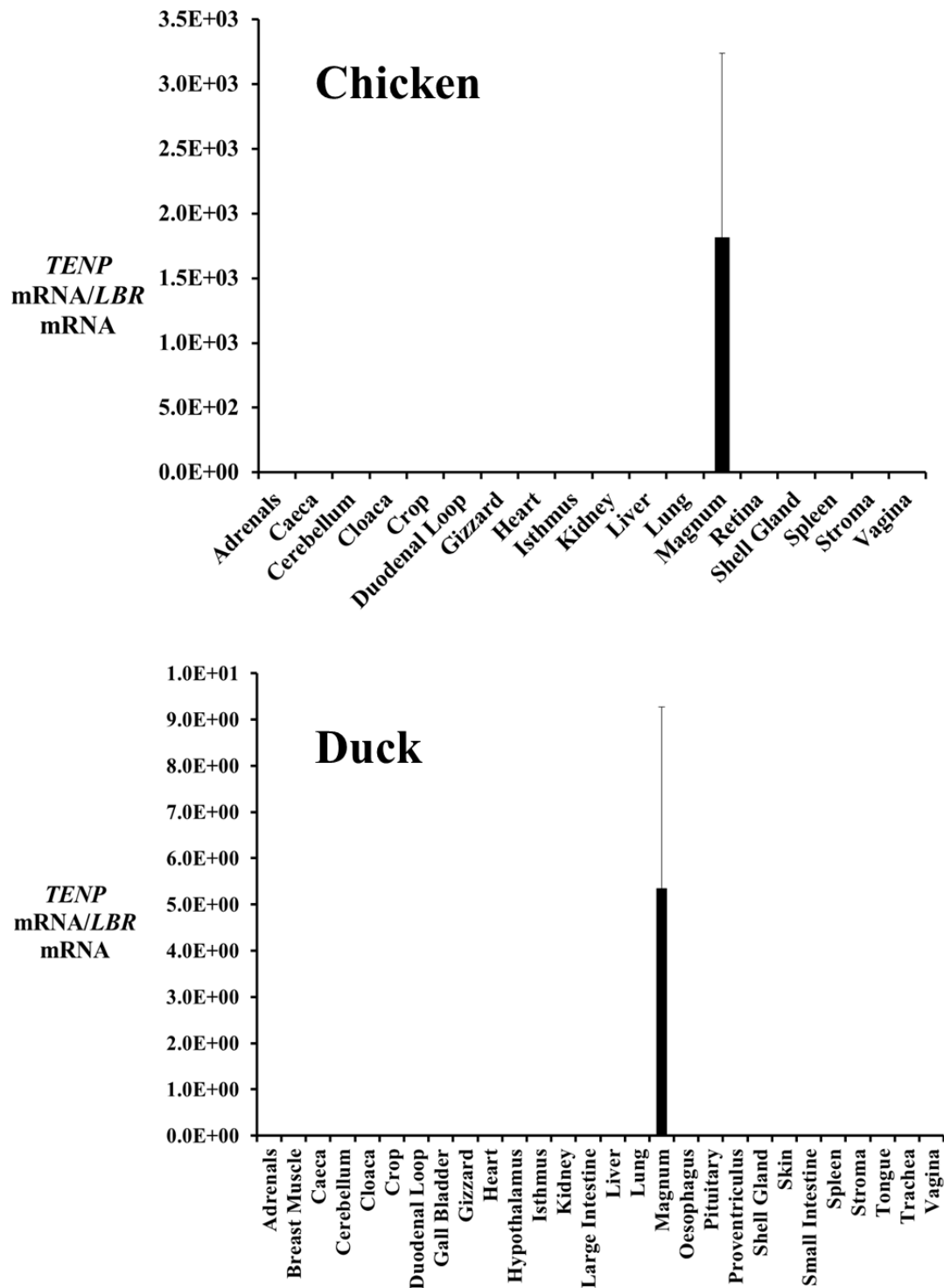


Figure 3.6: Expression of *TENP* mRNA in a range of adult chicken (n=4, mean \pm sem) and duck (n=3, mean \pm sem) tissues measured by RT-QPCR. *TENP* expression was corrected for chicken LBR and duck LBR expression to normalise for any differences between tissues. Expression is restricted to the oviduct in both species with the greatest level of expression seen in the magnum.

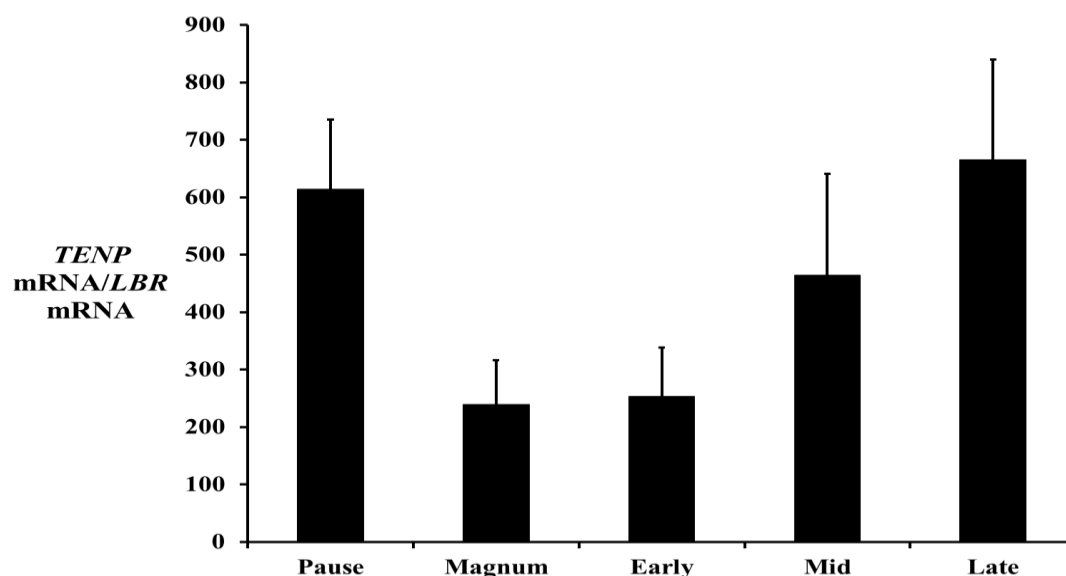


Figure 3.7: Expression of *TENP* mRNA in magnum tissue at different stages of egg formation measured by RT-QPCR (n= 8, mean ± sem). *TENP* expression was corrected for LBR expression. Pause represents a day when the hen did not ovulate so no egg is present. Magnum represents tissue when an egg is present in the magnum and early, mid and late describes the stage of shell formation in the shell gland and indicates the egg has left the magnum. ANOVA, P=0.083.

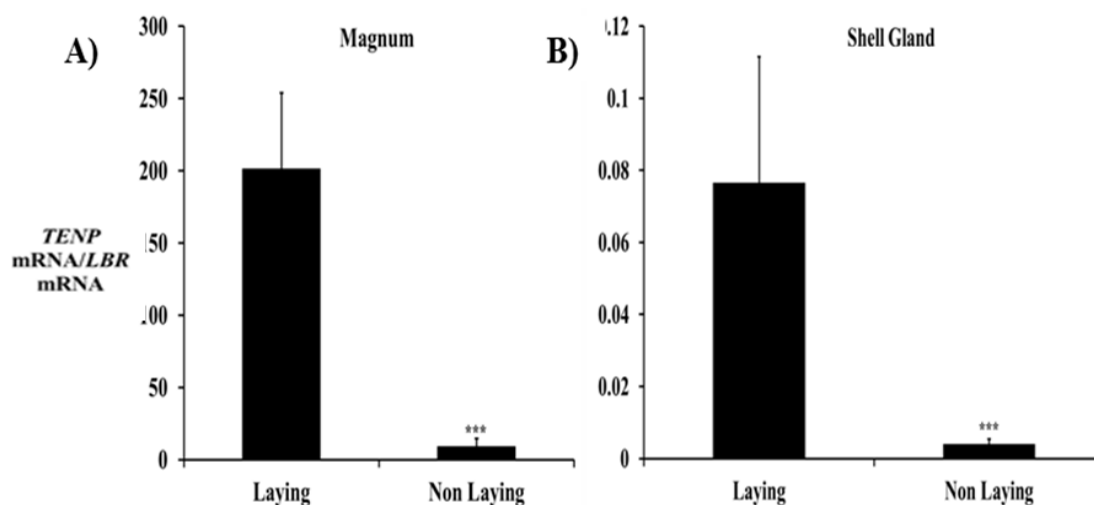


Figure 3.8: Expression of *TENP* mRNA in A) magnum and B) shell gland tissue of laying (L) and out of lay (NL) birds measured by RT-QPCR (n=11, mean ± sem). Non-laying hens were those where the oviduct had regressed due to the withdrawal of gonatrophic support with the onset of incubation behaviour. *TENP* expression was corrected using LBR expression. Note the large difference in the Y-axis scale between A and B. Significance between laying state is indicated at P<0.001, ***.

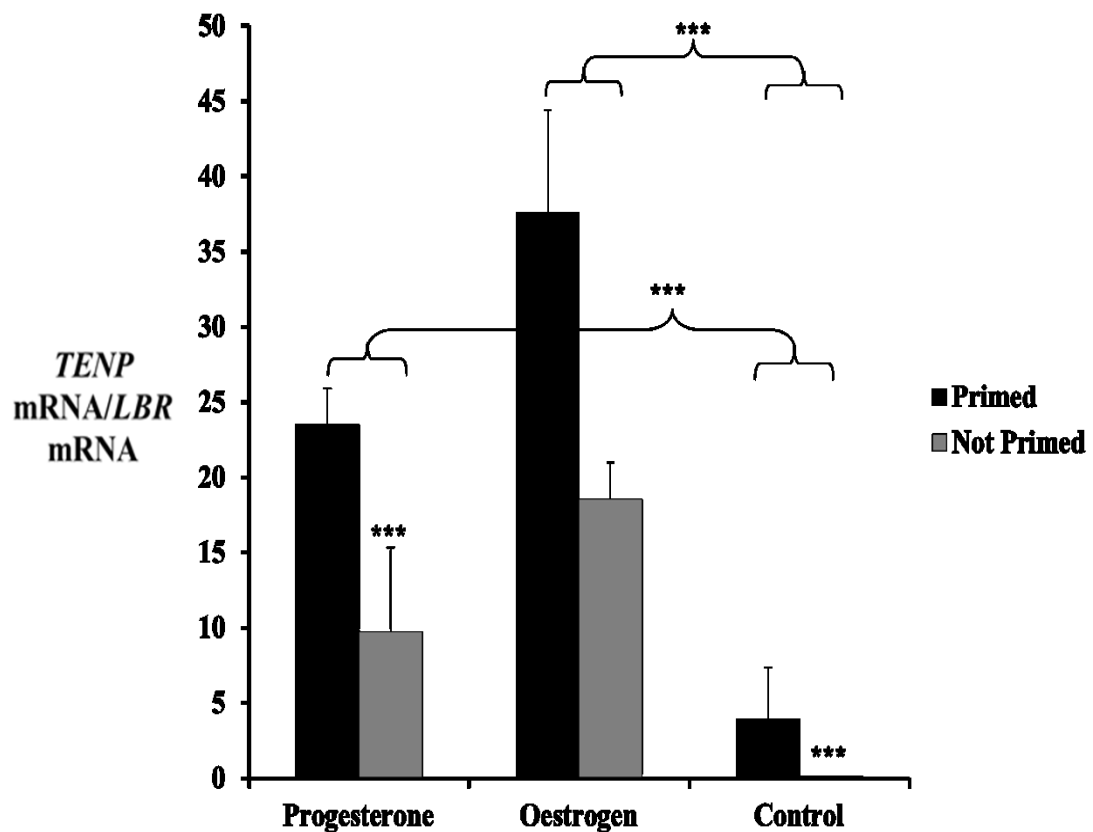


Figure 3.9: Expression of *TENP* mRNA in the magnum of juvenile chicks treated with steroids measured by RT-QPCR (n=10, mean \pm sem). *TENP* expression was corrected using LBR expression. Female chicks at 3 weeks of age were either primed with diethylstilbestrol or not primed (vehicle control) then subsequently treated with either progesterone, oestradiol (oestrogen) or vehicle (control). ANOVA indicated primed or not primed was significant at $P < 0.001$; steroid treatment was significant at $P < 0.001$. Significance between primed state within treatment is indicated, $P < 0.001$ (*) and significance between treatments regardless of primed state between the brackets, $P < 0.001$ (***)**.

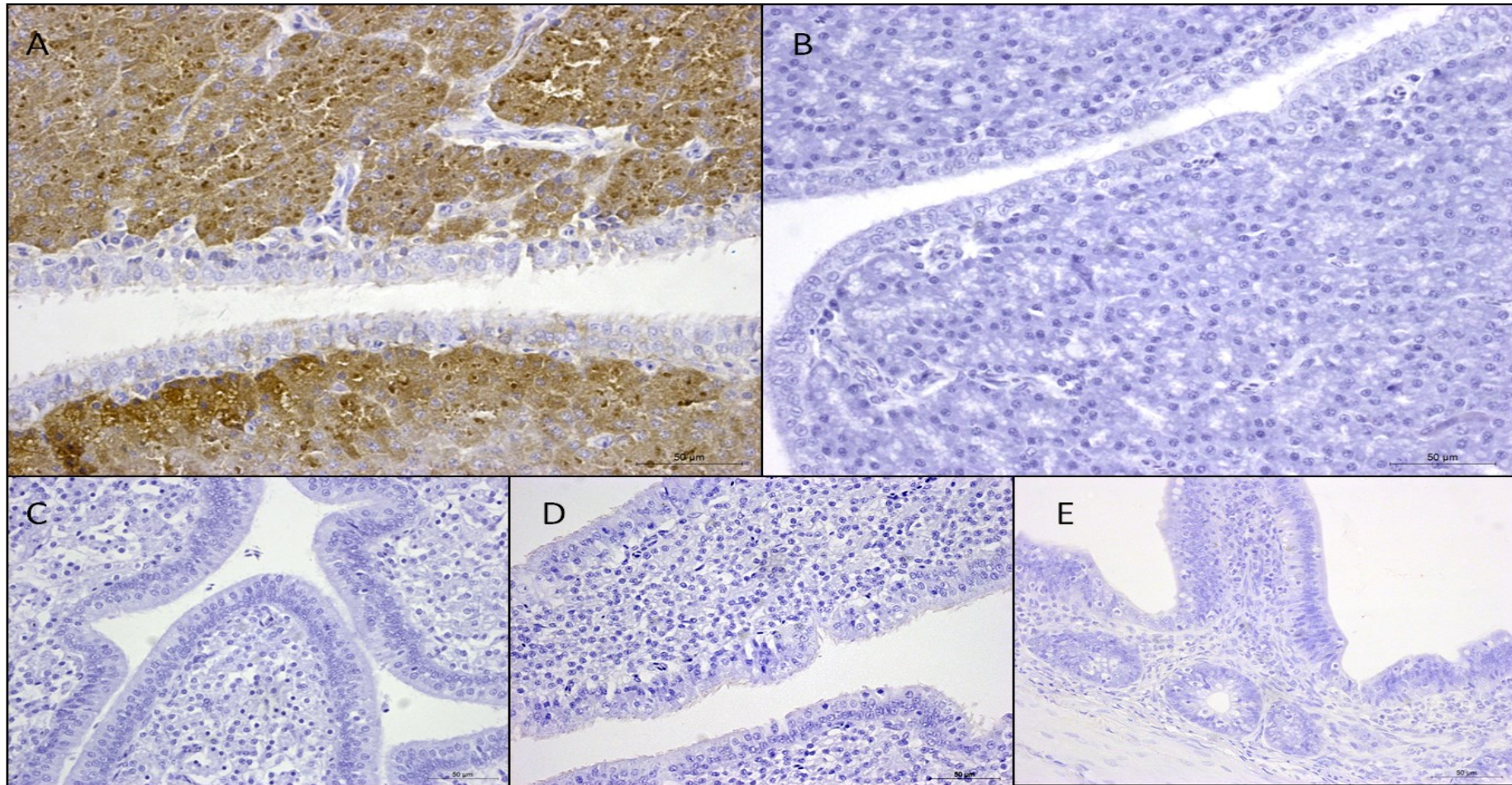


Figure 3.10: The tubular gland cells of the magnum region (A) of the oviduct stained positive and the surface epithelium (ciliated and non-ciliated cells) did not stain with anti-TENP antisera (107_AWM_1.1). The corresponding negative controls for A are shown in image B. The Isthmus (C), Shell gland (D) and Caecum (E) were not reactive to the primary antibody.

cannot be ruled out and it may serve both these roles. The fact that this protein also plays a transient role in the developmental stages (Yan and Wang, 1998), at least in the brain, of the chicken embryo suggests a dual purpose for TENP. This makes understanding how expression of *TENP* is controlled and its purpose extremely valuable in understanding the biology of not only the egg but the chick as well.

The EST data aligned to the May 2006 chicken genome assembly confirmed the expression of both of the *TENP* BPI domains in the hen reproductive tract but offered little evidence for their expression in one full length *TENP* transcript, as originally identified in neural precursors (<http://genome.ucsc.edu/>), rather supporting the conclusion that the protein may be expressed as two separate molecules each encoding for one of the BPI domains (Figure 3.3). Sequencing of PCR products using magnum cDNA with primers designed to amplify the whole of the *TENP* protein coding region produced a full length transcript including the new predicted translational start site and a second smaller transcript, which if translated would encode only for the BPI2 region of the TENP protein (Figure 3.2). Although in theory intensities of DNA bands on a gel amplified from targets with the same primers should be indicative of the relative abundance of each transcript in the starting sample if amplification efficiency is equal (Cottrez et al., 1994), factors such as amplicon size or sequence can lead to a bias in amplification with smaller amplicons being amplified more efficiently (Cha and Thilly, 1993) or possibly differences in reverse transcription efficiency. Indeed Northern analysis indicated that the full length transcript (Figure 3.1) was the dominant form of *TENP* and the lack of signal for the smaller transcript suggested that it may be below the threshold of detection for this method contrary to the PCR results. Although these results indicate that a transcript encoding both BPI-like domains is the dominant form in the oviduct the presence of a smaller transcript cannot be ruled out completely. It may be that this gene encodes for alternate forms of TENP where only one of the BPI domains is expressed, as seen with SPLUNCs (Bingle and Craven, 2002).

The PCR products from this study (TENP Exon1F1-Tenp End1) were identical to the available genomic and EST sequences yet contained an indel when compared to the

previously published *TENP* sequence (Yan and Wang, 1998) resulting in a frameshift (Figure 3.1). I therefore propose that the previously published protein sequence for TENP is either incorrect or specific to the strain used in that study. Potential *TENP* homologues were identified in both the duck and turkey genomes and sequencing of PCR products confirmed the presence of *TENP* mRNA in the magnum of both species. A potential *TENP* homolog is predicted in the zebra finch (NCBI Reference Sequence: XP_002192628.1) and TENP has also been identified in proteomic analysis of emu egg white (Maehashi et al., 2010). These results coupled with a high level of sequence similarity suggest that *TENP* is highly conserved across avian species. From the phylogenetic analysis (Figure 3.4) the *TENP* molecules are more similar to each other than either *Ovocalysin 36* or *LPLUNC2*. The analysis infers that *TENP* is a divergent ortholog of *LPLUNC2*, and that *Ovocalysin 36* may have evolved from an ancestral *LPLUNC2*-related gene as previously suggested (Chiang et al., 2011), perhaps from a duplication event occurring before mammals and birds diverged.

Since this analysis was carried out deposits in the GenBank database made by Kinoshita K., et al became visible (accession no. BAM13270.1, BAM13271.1, BAM13272.1, BAM13273.1, BAM13274.1 and BAM13275.1). The text proposed that TENP is identical to the chicken egg white protein ovoglobulinG2 and is expressed as a full length protein in the adult reproductive tract which would concur with the results seen in this study, however no paper has apparently been published relating to this observation. It is probable that these submissions refer to a globulin fraction separated by Longworth et al in 1940. In the paper Longworth et al identified three globulins G₁, G₂ and G₃ using electrophoresis to separate egg white and examined the electrophoretic patterns of ovalbumin and the globulin fractions.

Analysis of *TENP* sequence from the genome discovered a potential alternative translational start site which was confirmed as the dominant site used for transcription in magnum tissue. Because the expression of *TENP* is low in the embryo it was not possible to confirm the transcriptional start site. The proof from the magnum that the predominant translational start site is at position 32 (Figure

3.1C) combined with the Signal P predictions probability score of 0.996 for the new signal peptide probability and the signal peptide cleavage point suggest that at least in the adult the sequence proposed in this thesis is correct. There is no experimental evidence to support the translational start site, however, if we were to assume a typical Kozac initiation of translation (Kozak, 1999) then the evidence for the transcription start site from the 5' RACE makes the proposed translational start site the most likely. However it must be noted that in the absence of functional evidence the translational start site cannot be confirmed as upstream ATGs occur frequently in vertebrate mRNA (Rogozin et al., 2001) and the possibility of multiple sites of translation cannot be excluded (Kochetov et al., 2005).

Measurement of *TENP* expression in embryonic brain and retinal tissues concur with the developmental down-regulation seen by Yan et al (Yan and Wang, 1998); expression seen at E10 is dramatically decreased by E16 and no expression is detected in adult brain or retinal tissues (Figure 3.5). In contrast expression of *TENP* in the adult hen is restricted to the oviduct (Figure 3.6) suggesting at this stage *TENP* plays a different role. Expression is almost exclusively in the magnum with expression here around 400 x more than in the shell gland and almost 10,000x greater than in embryonic retina tissue. The magnum is responsible for the production of egg white and the high level of expression here would suggest that *TENP* is primarily a component of egg white. However, this study did detect some expression in other regions of the oviduct which would concur with the proteomic evidence for *TENP* in the other egg compartments e.g. the shell (Mann et al., 2006). Immunohistochemistry confirmed the presence of *TENP* as a protein in the tubular gland cells of the magnum region of the oviduct (Figure 3.10); this would support the secretion of *TENP*. Interestingly *TENP* was not detected in the epithelial cells which may indicate that *TENP* is less involved in local protection of the oviduct. In duck tissues *TENP* expression is also restricted to the magnum of the oviduct (Figure 3.6). *TENP* expression in the caecum had previously been reported by Chiang et al in 2011 (Chiang et al., 2011) however when using a quantitative approach no

significant expression was measured in this tissue compared to other tissues (Figure 3.6) which was further confirmed through IHC (Figure 3.10).

Expression in the magnum of the oviduct did not differ significantly in relation to the position of the egg in the oviduct at the time of sampling or if it was a pause day when no egg was present, although there was a tendency for the level to be lower when the egg was in the magnum or had recently left it ($P=0.051$) (Figure 3.7). This pattern of expression is typical of egg proteins and has been described previously for others including gallin (Gong et al., 2010) and ovalbumin (Muramatsu et al., 1994). In the magnum and shell gland there is significantly more *TENP* expression when the hen is in lay compared to when the oviduct is regressed (Figure 3.8) suggesting the expression of *TENP* is specifically up regulated during egg production when steroids are elevated and is likely to be under the control of gonadal steroids. This was confirmed by the measurement of *TENP* expression after the administration of oestrogen and progesterone to juvenile hens, with the increase in expression greatest with the dose of oestrogen compared to that of progesterone. Most importantly the response was strongest when the animals had previously been primed with an oestrogenic compound and showing that oestrogen and progesterone act synergistically as one would expect of an oviduct gene controlled directly or indirectly by gonadal steroids. Analysis for transcriptional factor binding sites 20kb upstream of the gene using MATCH (www.gene-regulation.com) did not identify any oestrogen or progesterone receptors so the effect may be secondary to stimulation of transcription factors by these steroids.

These results strongly suggest that in the adult hen *TENP*'s role is as a major component of the egg. However, local tissue activity, for example antimicrobial protection of the oviduct cannot be ruled out and it may serve both these roles. It is also notable that proteomic analysis has recently identified *TENP* as a possible requisite host protein in infectious bronchitis virus (IBV) life cycles (Kong et al., 2010b). The relationship of *TENP* to the PLUNC/BPI family of proteins suggests a role for *TENP* in the innate defence of eggs against pathogens and the presence of LPS binding domains would make an antimicrobial role seem possible as

demonstrated with Ovocalyxin-36 (Gautron et al., 2011). If this is the case it is interesting to note that TENP is present in all components of the egg and is a significant egg white component representing ~0.1-0.5% of the total protein, similar to the levels of ovoinhibitor, a multitype serine proteinase inhibitor which is present at 0.1-1.5% (Guerin-Dubiard et al., 2006). However since this study began antimicrobial function has been demonstrated with TENP isolated from emu egg where it is a large component (~15%) (Maehashi et al., 2014). In the emu egg there is very little lysozyme present and as such TENP is thought to play a large part in the defence of the egg. The large number of known antimicrobial proteins already identified in the egg and the activity of emu TENP would make an antimicrobial role for chicken TENP seem plausible although experimental proof in the chicken does not yet exist.

Despite the fact that chicken TENP is likely to be an antimicrobial agent it was not chosen as a candidate for feed trials due to the difficulties producing it in large quantities in our fungal expression system (see chapter 5). In order for a protein to be a successful alternative to existing in-feed antibiotics it needs to be produced in large quantities, cheaply, otherwise it cannot be considered economically viable. In addition to TENP being difficult to produce in our system the fact that it is a large protein makes it susceptible to enzymatic protein cleavage. As the proposed strategy for peptide administration is in the feed the antimicrobial candidate must be able to withstand digestion in the intestine of the target organism. One of the main enzymes in the gastrointestinal tract is pepsin, an *in-silico* digestion of the mature TENP protein revealed 136 pepsin cleavage sites at pH>2, focus was therefore shifted to the smaller peptides in this study, the ovodefensins. However it is not to say that it might have other applications as an antimicrobial.

Chapter 4

Expansion and characterisation of the ovodefensin family

4.1. Introduction

The egg has many mechanisms in place to protect itself from bacterial invasion; these can effectively be considered as either physical or chemical (Kovacs-Nolan et al., 2005). An important part of the eggs chemical defence is provided by antimicrobial peptides (AMPs), otherwise known as host defence peptides (HDPs). A particular group of these, the ovodefensins, was recently shown to be a new family of egg specific defensins (Gong et al., 2010). The family had been shown to be conserved across divergent avian species and was thought to be avian specific (Gong et al., 2010). Proteomic methods had confirmed the presence of the chicken, turkey and duck members of the ovodefensin family in the egg (Odani et al., 1989, Mann, 2007, Naknukool et al., 2008) and quantitative reverse transcriptase PCR confirmed that the expression of the chicken member, *gallin* was restricted to the oviduct of the hen (Gong et al., 2010). In contrast classical defensins are widely distributed across many tissues and can be found in all vertebrates (van Dijk et al., 2008). Only three of the classical chicken defensins are found in the egg; AvBD9, 10 and 11 (Herve et al., 2014). It remained to be determined if the expression of other avian members of the ovodefensin family are also restricted to the oviduct, which would imply that the whole family was likely to be expressed principally for inclusion in the egg. If this is the case ovodefensins are likely to be influenced by gonadal steroids and would be expected to show expression patterns in response to steroids typical of egg specific genes such as ovalbumin (Muramatsu et al., 1994) or TENP (Whenham et al., 2014). Although the connection had not previously been made with classical defensins, ovodefensins were classed as a new branch of this family largely because of the conservation of a characteristic 6 cysteine motif, linked by 3 disulphide bonds and a common glycine residue attributed to all classical β -defensins (Gong et al., 2010). Their position in the genome is also close to the β -defensin cluster on chromosome 3 (Gong et al., 2010). The ovodefensins differ from classical defensins in the spacing of amino acids within the 6 cysteine motif, and are slightly shorter in length, the mature peptides ranging from only 39-41 amino acids, although they are still highly cationic as is expected with defensins. Two cysteine motifs were observed in ovodefensins; C-X5-C-X3-C-X11-C-X3-CC and C-X3-C-X3-C-X11-C-

X4-CC (Gong et al., 2010), which may be due to the fact that antimicrobial peptides are often under high selective pressure to evolve due to the ongoing arms race between pathogen and host, such as observed in classical β -defensins (Maxwell et al., 2003). Interestingly the 3D structure of the chicken ovodefensin gallin has recently been solved which confirmed the presence of the three-stranded antiparallel β -sheet observed in all classical β -defensins reinforcing its relationship with the β -defensins (Herve et al., 2014). However, gallin contains an additional short two-stranded β -sheet (Herve et al., 2014), this five-stranded arrangement supports the hypothesis that gallin, and presumably the other ovodefensins, form a structurally distinct sub-family of β -defensins.

Due to an increase in antibiotic resistance there is an ever increasing need to find alternative strategies for microbial control. Host defence peptides such as the defensins have been suggested previously as an interesting template for new classes of antimicrobial drugs as they often possess a wide spectrum of antimicrobial activity (Hancock, 1997, Hancock and Lehrer, 1998). Cationic host defence peptides are small, typically containing a high abundance of positively charged and hydrophobic residues (Hancock and Sahl, 2006). They have been studied not only for their direct antimicrobial activities but also for multifaceted immunomodulatory capabilities (Hancock and Sahl, 2006). As it is hoped that host defence peptides such as ovodefensins may provide a new source of novel antimicrobials it is important to broaden our understanding of their biology and hopefully discover new molecules which may possess antimicrobial function.

4.2. Objectives

The aim of this chapter was to test the hypothesis that evolution is acting on spacing as well as sequence which may reveal the presence of further cysteine motifs and new molecules of the ovodefensin family in databases or the genomic sequences. The aim was to determine whether the family is avian specific as previously speculated. This chapter will also examine in greater detail the expression of the ovodefensin family both in terms of tissue, start site and steroid control.

4.3. Methods

4.3.1. Discovery of new ovodefensin family members

Available genome databases Ensembl (<http://www.ensembl.org>), PreEnsembl (<http://pre.ensembl.org>) and UCSC (<http://genome.ucsc.edu/>) were searched using TBLASTN and BlastP to locate potential homologs using the 41 amino acid mature peptide sequence of gallin (GenBank: CBE70283.1) and the previously published 39 amino acid mature peptide sequence of taeniopygin 2 (Gong et al., 2010). Further iterative searches were made with the homologues discovered. Protein database UniProt (<http://www.uniprot.org/>) was also searched using BlastP to identify peptide sequences previously unidentified as ovodefensins.

4.3.2. Phylogenetic analysis of the evolution of spacing between conserved residues.

A distance matrix based on the amino acid sequence length between each of the cysteines and the conserved glycine residue was built for analysis by R for all known and newly discovered ovodefensins (Table 4.1). Hierarchical clustering for each distance matrix was calculated using R (<http://www.r-project.org/>) which was then used to produce a cladogram of the phylogenetic relationships using the R ‘hclust’ function (Figure 4.1A). Similarly cladograms based on spacing were produced separately for avian species (Figure 4.1C) and reptiles (Figure 4.1D). The resulting ovodefensin ‘sub-families’ were used to propose new nomenclature for existing and newly discovered ovodefensin molecules (Table 4.2) which currently have trivial names based on a range of criteria determined by the discoverer. I propose that each gene has the prefix OvoD to identify it as an ovodefensin and is attributed a letter from A-F to identify the sub-family to which it belongs. Within a sub-family each gene is given a numerical identifier allowing multiple forms of the same gene to be identified. For example *gallin* would become *Gallus gallus OvoDA1* and the additional copies *OvoDA1_2* and *OvoDI_3*. Through the use of this nomenclature *meleagrin* is *Meleagris gallopavo OvoDA1* allowing it to be easily identified as a

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	
1	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
2	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
3	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
4	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
5	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
6	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
7	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
8	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
9	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
10	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
11	0	0	0	0	0	0	0	0	0	0	0	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	1	1	3.61	3.61	3.61	3.61	3.61	3.74	3.74	3.74
12	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
13	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
14	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
15	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
16	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
17	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
18	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
19	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
20	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
21	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
22	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	0	0	0	0	0	0	0	0	0	0	0	0	2.83	2.83	2.83	2	2	1.41	1.41	1.41	1.41	1.41	1.73	1.73	1.73
23	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	0	0	0	2	2	3.74	3.74	3.74	3.74	3.74	3.87	3.87	3.87
24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	0	0	0	2	2	3.74	3.74	3.74	3.74	3.74	3.87	3.87	3.87
25	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.24	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	0	0	0	2	2	3.74	3.74	3.74	3.74	3.74	3.87	3.87	3.87
26	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	3.16	3.16	3.16	3.16	3.16	3.32	3.32	3.32
27	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	3.16	3.16	3.16	3.16	3.16	3.32	3.32	3.32
28	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	3.74	3.74	3.74	3.16	3.16	0	0	0	0	0	1	1	1
29	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	3.74	3.74	3.74	3.16	3.16	0	0	0	0	0	1	1	1
30	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	3.74	3.74	3.74	3.16	3.16	0	0	0	0	0	1	1	1
31	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	3.74	3.74	3.74	3.16	3.16	0	0	0	0	0	1	1	1
32	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41	3.74	3.74	3.74	3.16	3.16	0	0	0	0	0	1	1	1
33	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	3.87	3.87	3.87	3.32	3.32	1	1	1	1	1	0	0	0
34	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	3.87	3.87	3.87	3.32	3.32	1	1	1	1	1	0	0	0
35	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	3.74	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	3.87	3.87	3.87	3.32	3.32	1	1	1	1	1	0	0	0

Table 4.1: Distance matrix calculated from the inter cysteine and conserved glycine residues of all known Ovoidensin homologs using R. Numbers 1-35 on each axis represent each of the Ovoidensin molecules; the numbers within the table represent the distance between each Ovoidensin spacing sequence. Colour indicates the similarity of spacing between peptides 1-35 on the x and y-axis with green representing no difference and red those with the greatest difference.

Species	Latin name	Gene/ protein name	Mature peptide sequence
Chicken	<i>Gallus gallus</i>	OvoDA1	LVLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLPASW KWK
Chicken	<i>Gallus gallus</i>	OvoDA1_2	LVLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLPASW KWK
Chicken	<i>Gallus gallus</i>	OvoDA1_3	LVLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLPASW KWK
Chicken	<i>Gallus gallus</i>	OvoDB1	KRKGTCKGYCAPTCNKKDEWSFHQSCKKMYCCLPLK KGK
Turkey	<i>Meleagris gallopavo</i>	OvoDA1	QVLKYCPKIGYCSSKCSKAENVWAYSPDCKVHCCVPANQ KWK
Turkey	<i>Meleagris gallopavo</i>	OvoDA1_2	QVLKYCPKIGYCSSKCSKAENVWAYSSDCKVHCCVPANR KWK
Turkey	<i>Meleagris gallopavo</i>	OvoDB1	QHKGTCKGYCASACNKKDEWTFHQSCCKMYCCLPPPK KGK
Duck	<i>Anas platyrhynchos</i>	OvoDA1	QVRKYCPKVGYSKCSKADVWSLSSDCKFYCCLPPGW KGK
Duck	<i>Anas platyrhynchos</i>	OvoDB1	QKKGFCAGYCSYCAKTDEWTFHQTCGKMYCCIPPPKK GK
Zebra finch	<i>Taeniopygia guttata</i>	OvoDA1	KFRKTCAPMGYCSPKCRVMDLKYTSGDCKYSCCIPTAW KGK
Zebra finch	<i>Taeniopygia guttata</i>	OvoDA1_2	KFRKTCAPMGYCSPKCRVMDLKYTSGDCKYSCCIPTAW KGK
Zebra finch	<i>Taeniopygia guttata</i>	OvoDB1	QPKRSCRGHCSRTCCKGEREEHTEDCGGMHCCLTHRRK K
Rock pigeon	<i>Columba livia</i>	OvoDA1	QVKKYWTYCPKEGHCSSKCDKMYTWTSSDCKFYCCIP YSWNGK
Rock pigeon	<i>Columba livia</i>	OvoDB1	QYKGVCPGYCSNMCDKMDWGFSSRSCRKMYCCIPPLK KGK
Black swan	<i>Cygnus atratus</i>	OvoDA1	QVRKYCPKVGYSKCSKADVWSLSSDCKFYCCLPPGW K
Budgerigar	<i>Melopsittacus undulatus</i>	OvoDA1	QFKGSCAGYCSHMAKRDWTFHSCGKMYCCIPPPKK GK
Collared flycatcher	<i>Ficedula albicollis</i>	OvoDA1	IRKTCAPVGYCSPKCNVLDLKYTSADCKYSCCIPNPWK K
Collared flycatcher	<i>Ficedula albicollis</i>	OvoDB1	FCDGYCAHACGETEWSFSPYCEELHCCIPSPKKKGK
Medium ground finch	<i>Geospiza fortis</i>	OvoDA1	VRKTCAPVGYCSPKCRVMDLKYTSADCKYSCCIPTSWK GK
Medium ground finch	<i>Geospiza fortis</i>	OvoDA1_2	VRKTCAPVGYCSPKCRVMDLKYTSADCKYSCCIPTSWK GK
Medium ground finch	<i>Geospiza fortis</i>	OvoDA1_3	VRKTCAPVGYCSPKCRVMDLKYTSADCKYSCCIPTSWK GK
Loggerhead turtle	<i>Caretta caretta</i>	OvoDB1	EKKCPGRCTLKCGKHERPTLPYNCCKYICCVPVKVK
Chinese turtle	<i>Pelodiscus sinensis</i>	OvoDB1	KGCAGRCDWKCCKGYEYETTYDCPKSYCCLPLFKWK
Chinese turtle	<i>Pelodiscus sinensis</i>	OvoDB1_2	KGCAGRCDWKCCKGYEYETTYDCPKSYCCLPLFKWK
Painted turtle	<i>Chrysemys picta bellii</i>	OvoDB1	KGGCPGRCAKSCDKHEVASKTFDCKYMKCCTYPKKG
Painted turtle	<i>Chrysemys picta bellii</i>	OvoDC1	QKKGCPGRCTSKCGKYEYPSKITYPNCKTLCCIASKRGK
Painted turtle	<i>Chrysemys picta bellii</i>	OvoDC1_2	QKKGCPGRCTSKCGKYEYPSKITYPNCKTLCCIASKRGK
Painted turtle	<i>Chrysemys picta bellii</i>	OvoDC2	QKKGCPGRCTSKCGKYEYPSKITYPNCKALCCISSKKKGK
Anole Lizard	<i>Anolis carolinensis</i>	OvoDE1	KPSCSVDGGRCAYCEKTEYASYVHDCPLCCFKYKK
Anole Lizard	<i>Anolis carolinensis</i>	OvoDE2	KPSCSVDGGRCAYCEKTEYASYVHNCPLCCFKYKK
Anole Lizard	<i>Anolis carolinensis</i>	OvoDE2_2	KPSCSVDGGRCAYCEKTEYASYVHNCPLCCFKYKK
Anole Lizard	<i>Anolis carolinensis</i>	OvoDE2_3	KPSCSVDGGRCAYCEKTEYASYVHNCPLCCFKYKK
Anole Lizard	<i>Anolis carolinensis</i>	OvoDE3	KPSCSVDGGRCAYCEKTEYGYGYDCPLCCFKYKK
Anole Lizard	<i>Anolis carolinensis</i>	OvoDF1	KGPSCSTYGGSCNPKGCAKGYTSYTHDCKYCCVSP
Anole Lizard	<i>Anolis carolinensis</i>	OvoDF2	KGPSCKEYGGICNPKSCAKGYSSYSTYDCKTCC
Anole Lizard	<i>Anolis carolinensis</i>	OvoDF3	KGPSCSTYGGSCNPKGCAKGYTSYTHDCKYCCVSP
American alligator	<i>Alligator mississippiensis</i>	OvoDD1	PKEPCGGRCVKKCDKDEVASSTYDCKFLCCTRPKKRQ
American alligator	<i>Alligator mississippiensis</i>	OvoDD2	PKEPCGGRCVKKCDKGEVASSTYDCKLLCCTRPKKR

Table 4.2 - All known and newly discovered ovodefensins

New gene/peptide name	Species	Previous definition	First defined	Accession no.	Genome location
OvoDA1	<i>Gallus gallus</i>	Gallin_1	(Mann, 2007)	GenBank CBE702(78-83)	Chr3; 106740601-106740723
OvoDA1_2	<i>Gallus gallus</i>	Gallin_2	(Mann, 2007)	GenBank CBE702(78-83)	Chr 3; 106748086-106748208
OvoDA1_3	<i>Gallus gallus</i>	Gallin_3	(Mann, 2007)	GenBank CBE702(78-83)	Chr 3; 106751375-106751497
OvoDA1	<i>Meleagris gallopavo</i>	Meleagrin	(Odani et al., 1989)	UniProtKB/Swiss -Prot: P21376.2	Chr 2; 112906487-112907670
OvoDA1	<i>Cygnus atratus</i>	Cygnin	(Simpson and Morgan, 1983)	UniProtKB/Swiss -Prot: P02785.3	no genome
OvoDA1	<i>Anas platyrhynchos</i>	dBPS1	(Naknukool et al., 2008)	UniProtKB/Swiss -Prot: P85123.1	Scaffold KB746044.1; 6812-6934
OvoDB1	<i>Anas platyrhynchos</i>	dBPS2	(Naknukool et al., 2008)	UniProtKB/Swiss -Prot: P85124.1	Scaffold KB746044.1; 12527-12649
OvoDA1	<i>Taeniopygia guttata</i>	Taeniopygin 1	(Gong et al., 2010)	EMBL: LN717251	Chr 3; 111190828-111191187
OvoDA1_2	<i>Taeniopygia guttata</i>	Taeniopygin 1_2	(Gong et al., 2010)	EMBL: LN717252	Chr 3; 111176525-111176647
OvoDB1	<i>Taeniopygia guttata</i>	Taeniopygin 2	(Gong et al., 2010)	EMBL: LN717253	Chr 3; 111193797-111194424
OvoDA1	<i>Columba livia</i>	Cygnin	(Shapiro et al., 2013)	GenBank EMC87474.1	no genome
OvoDB1	<i>Columba livia</i>	Small basic protein 1	(Shapiro et al., 2013)	GenBank: EMC87475.1	no genome
OvoDB1	<i>Caretta caretta</i>	TEWP	(Chattopadhyay et al., 2006)	UniProtKB/Swiss -Prot: P0CAP0.1	no genome

Table 4.3 - Nomenclature of previously identified ovodefensins.

member of the same sub-family and a *gallin* ortholog. This nomenclature will be used throughout the remainder of this thesis to aid clarity.

In addition to this a cladogram was constructed using the programme Mega5 and the core peptide sequence from the conserved glycine until the fourth cysteine residue inclusively for sequences where the number of amino acids within this region was identical (Figure 4.1B). Mega5 was also used to construct phylograms based on the whole mature peptide sequences for OvoDA family members (Figure 4.1E) and also OvoDB (Figure 4.1F).

4.3.3. Animals and tissue collection

To determine *Gallus gallus* *OvoDA1* and *OvoDB1* expression tissues were taken from sexually mature White Leghorn LSL hens (Lohmann) (see 2.4.2). For analysis of *Anas platyrhynchos* *OvoDB1* (*dBPS₁*) and *OvoDA1* (*dBPS₂*) expression tissues were collected from sexually mature Pekin ducks (Cherry Valley) (2.4.4). *Combined expression of Meleagris gallopavo OvoDA1 (Meleagrin)* and *OvoDA2* and expression of *OvoDB1* was determined using tissues from sexually mature turkeys (2.4.3). *Taeniopygia guttata OvoDA1 (Taeniopygin 1)* and *OvoDB1 (Taeniopygin 2)* expression was measured using adult female zebra finches courtesy of Dr Karen Spencer, University of St Andrews, Scotland (see 2.4.5).

In order to assess the effect of oviposition on *Gallus gallus* *OvoDB1* expression magnum tissue was obtained from sexually mature White Leghorn hens with an ovum at various positions in the oviduct, see section 2.4.7 for details.

Gallus gallus *OvoDA1* and *OvoDB1* expression was measured in hens that were in lay (n=11) and those where the oviduct had regressed due to incubation behaviour (n=11) to establish the effect of oviduct development, (section 2.4.8).

4.3.4. RNA preparation

RNA was extracted from tissues and processed as per the Ultraspec protocol (section 2.5.1).

Gene/peptide name	Species	Accession no.	Genome build/ location
OvoDB1	<i>Gallus gallus</i>	EMBL: LN717248	Nov'11; Chr 3; 106744444-106744572
OvoDA1_2	<i>Meleagris gallopavo</i>	EMBL: LN717249	Dec'09; Chr 2; 112968507-112968850
OvoDB1	<i>Meleagris gallopavo</i>	EMBL: LN717250	Dec'09; Chr 2; 112960926-112961582
OvoDA1	<i>Melopsittacus undulatus</i>	-	melUnd1; Scaffold JH556573; 2958896-2959015
OvoDB1	<i>Pelodiscus sinensis</i>	-	PelSin_1.0; Scaffold JH212527; 3638-3745
OvoDB1_2	<i>Pelodiscus sinensis</i>	-	PelSin_1.0; Scaffold JH210373; 9969-10076
OvoDB1	<i>Chrysemys picta bellii</i>	-	Dec'11; Scaffold JH584573; 2758224-2758331
OvoDC1	<i>Chrysemys picta bellii</i>	-	Dec'11; Scaffold JH584573; 2800433-2800549
OvoDC1_2	<i>Chrysemys picta bellii</i>	-	Dec'11; Scaffold JH584573; 2828206-2828322
OvoDC2	<i>Chrysemys picta bellii</i>	-	Dec'11; Scaffold JH584573; 2778988-2779104
OvoDE1	<i>Anolis carolinensis</i>	-	May'10; Scaffold Un_GL343551; 118450-118557
OvoDE2	<i>Anolis carolinensis</i>	-	May'10; Scaffold Un_GL343551; 106991-107098
OvoDE2_2	<i>Anolis carolinensis</i>	-	May'10; Scaffold Un_GL343551; 131862-131969
OvoDE2_3	<i>Anolis carolinensis</i>	-	May'10; Scaffold Un_GL343551; 142606-142713
OvoDE3	<i>Anolis carolinensis</i>	-	May'10; Scaffold Un_GL343551; 53724-53831
OvoDF1	<i>Anolis carolinensis</i>	-	May'10; Scaffold Un_GL343551; 64537-64644
OvoDF2	<i>Anolis carolinensis</i>	-	May'10; Scaffold Un_GL343553; 4868-4966
OvoDF3	<i>Anolis carolinensis</i>	-	May'10; Scaffold Un_GL343551; 60162-60269
OvoDD1	<i>Alligator mississippiensis</i>	-	Aug'12; Scaffold JH736037; 19230-19340
OvoDD2	<i>Alligator mississippiensis</i>	-	Aug'12; Scaffold JH736037; 4956-5063
OvoDA1	<i>Ficedula albicollis</i>	-	FicAlb_1.4; Scaffold JH603338; 1433524-1433405
OvoDB1	<i>Ficedula albicollis</i>	-	FicAlb_1.4; Scaffold JH603338; 1441614-1441721
OvoDA1	<i>Geospiza fortis</i>	-	Apr'12; Scaffold JH760839; 55-174
OvoDA1_2	<i>Geospiza fortis</i>	-	Apr'12; Scaffold JH739967; 2617679-2617798
OvoDA1_3	<i>Geospiza fortis</i>	-	Apr'12; Scaffold JH739967; 2632098-2632217

Table 4.4 – Nomenclature and genome location of newly identified ovodefensins.

Common Name	Latin Name	Abbreviation
Chicken	<i>Gallus gallus</i>	Gg
Turkey	<i>Meleagris gallopavo</i>	Mg
Duck	<i>Anas platyrhynchos</i>	Ap
Zebra finch	<i>Taeniopygia guttata</i>	Tg
Black swan	<i>Cygnus atratus</i>	Ca
Rock Pigeon	<i>Columba livia</i>	Cl
Budgerigar	<i>Melopsittacus undulatus</i>	Mu
Collared flycatcher	<i>Ficedula albicollis</i>	Fa
Medium ground finch	<i>Geospiza fortis</i>	Gf
Painted turtle	<i>Chrysemys picta bellii</i>	Cpb
Loggerhead turtle	<i>Caretta caretta</i>	Cc
Chinese soft-shell turtle	<i>Pelodiscus sinensis</i>	Ps
Anole lizard	<i>Anolis carolinensis</i>	Ac
American alligator	<i>Alligator mississippiensis</i>	Am

Table 4.5 - A key for the abbreviations of the species Latin name.

4.3.5. Transcript detection and characterisation

4.3.5.1. RT-PCR and sequencing

1 µg samples of chicken, duck, turkey and zebra finch magnum RNA were reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, UK) according to the manufacturer's protocol. Primers (Chapter 2, Table 2.2) were designed to ensure complete coverage of each ovodefensin's coding sequence. PCR amplification was performed using standard conditions (section 2.6.2). Amplified PCR fragments were sequenced with the forward and reverse primers. Sequences were assembled by Staden (Staden, 1996) to produce consensus sequences.

4.3.5.2. 5'/3' RACE

5'/3'RACE (Roche Diagnostics 2nd Generation, Mannheim, Germany) were carried out to determine the number of exons encoding *Gallus gallus OvoDA1* (*gallin*) (section 2.8). For 5'RACE primer OvoDA1SP2 was used for both amplification and sequencing. For 3'RACE, OvoDA1SP5 was used for amplification and sequencing. Primer sequences for RACE can be found in Chapter 2, Table 2.2.

4.3.6. Reverse transcription quantitative polymerase chain reaction (RT-QPCR).

0.5 µg of cDNA was prepared (section 2.5.2) and used for RT-QPCR analysis as detailed in section 2.7.2. Primer3 was used to design primers for quantification, sequences can be found in Chapter 2, Table 2.2. Reactions containing no template were run as a control. Lamin B-receptor (LBR) expression was measured in the same way to normalise concentrations (as described previously) (McDerment et al., 2012, Whenham et al., 2014) in order to determine the absolute concentration of the different ovodefensin transcripts. One way or two way ANOVA and least significant difference to test between the means were used as appropriate for statistical analysis of log transformed data. (Genstat 13th edition, VSN International Ltd, Oxon, UK).

4.3.7. Production and titres of polyclonal antibodies

Production of antibodies was carried out by Dundee Cell Products Ltd, Dundee (section 2.11); titres of anti-GalOvoDB1 and anti-TaeOvoDB1 in the antisera were measured (section 2.11) for use in immunohistochemistry.

4.3.8. Immunohistochemistry (IHC)

Wax embedded tissues were prepared (section 2.12), a standard IHC protocol was then used; optimal staining was achieved at a 1:500 dilution for the polyclonal zebra finch anti-OvoDB1 (113_KGE_2.1) and 1:1000 for the chicken anti-OvoDB1 (108 CNK-1.3).

4.3.9. Sequencing and database submission

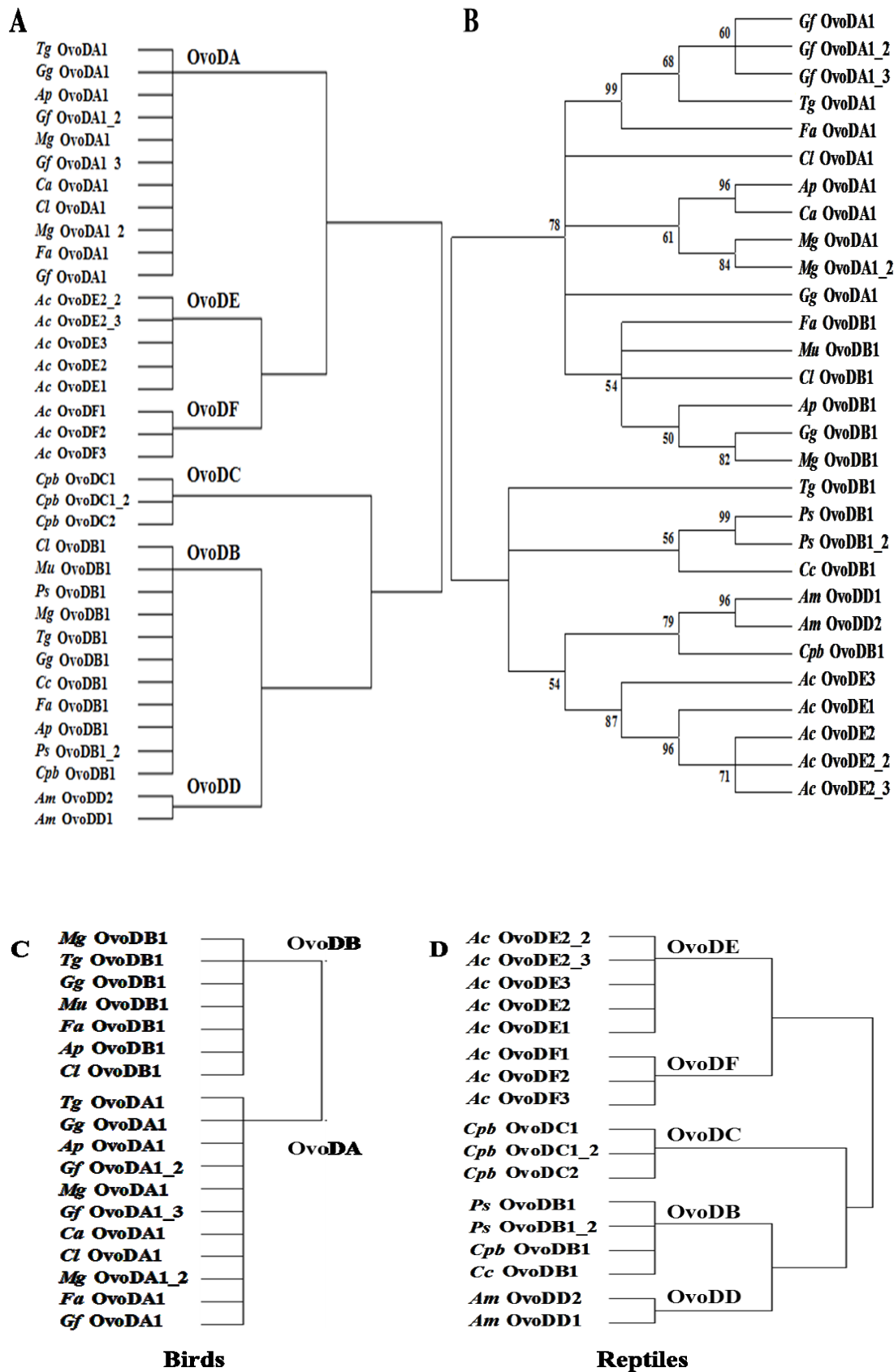
All sequencing was carried out by GATC biotech (Konstanz, Germany) and consensus sequences were submitted to EMBL, *Gallus gallus OvoDB1* (EMBL accession no. LN717248), *Meleagris gallopavo OvoDAI_2* (EMBL accession no. LN717249), *Meleagris gallopavo OvoDB1* (EMBL accession no. LN717250), *Taeniopygia guttata OvoDAI* (EMBL accession no. LN717251), *OvoDAI_2* (EMBL accession no. LN717252) and *OvoDB1* (EMBL accession no. LN717253). Putative budgerigar, medium ground finch, anole lizard, american alligator, collared flycatcher, painted turtle and chinese soft-shelled turtle sequences were not submitted because they remain predicted but can be found in Table 4.2.

4.4. Results

4.4.1. Bioinformatic analysis and transcript confirmation

TBLASTN similarity searches of available genomes located twenty four new ovodefensin homologues (Table 4.2, Table 4.4). Hierarchical clustering based on the distance between each cysteine identifies six specific sub-families termed OvoDA-OvoDF (Figure 4.1A). In the chicken the first representative of OvoDB, a shorter cysteine motif (C-X3-C-X3-C-X11-C-X4-CC) was identified on chromosome 3, where *OvoDAI* (C-X5-C-X3-C-X11-C-X3-CC) and the classical beta defensins are also located. This was named *OvoDB1* in accordance with the proposed

nomenclature outlined in 4.3.2 (Figure 4.1A, C, Table 4.3). The first turkey representative of the OvoDB1 motif (Figure 4.1A, C, Table 4.3) was discovered on chromosome 2 the same chromosome as *Meleagris gallopavo OvoDA1* (*meleagrin*). A further potential paralog of *Meleagris gallopavo OvoDA1* was also located on this chromosome; the mature peptide sequence shares a 95% identity with *OvoDA1* and was named *Meleagris gallopavo OvoDA2* (Figure 4.1A, C, Table 4.4). A budgerigar representative of sub-family B, *Melopsittacus undulatus OvoDB1* (Figure 4.1A, C, Table 4.4) was discovered during this analysis however a representative from the same subfamily (A) as *gallin* has yet to be located for this species. Three putative copies of a sub-family A ovodefensin were found in the April 2012 assembly of the medium ground finch genome; *Geospiza fortis OvoDA1*, *OvoDA1_2* and *OvoDA1_3* (Figure 4.1A, C, Table 4.4). A search of the flycatcher genome discovered a member from both the A and B sub-families, these were named *Ficedula albicollis OvoDA1* and *OvoDB1* respectively (Figure 4.1A, C, Table 4.4). For the first time reptile representatives of the ovodefensin family were identified. Two copies of *Pelodiscus sinensis OvoDB1* (Figure 1A, D, Table 4) were identified in the Chinese soft-shell turtle genome. In the painted turtle genome a sub-family B member was also located; *Chrysemys picta bellii OvoDB1* (Figure 1A, D, Table 4) in addition to this a new cysteine spacing motif (C-X3-C-X3-C-X13-C-X3-CC), which we have termed sub-family C was uncovered. Within this the painted turtle sub-family C contains two copies of the *OvoDC1* gene and an *OvoDC2* gene (Figure 4.1A, D, Table 4.4). TBLASTN searches of the American alligator genome identified two members of yet another new cysteine motif, sub-family D (C-X3-C-X3-C-X11-C-X3-CC); *Alligator mississippiensis OvoDD1* and *OvoDD2* (Figure 1A, D, Table 4). Finally two further motifs were uncovered in the anole lizard genome; sub-family E (C-X6-C-X3-C-X11-C-X2-CC) and F (C-X6-C-X4-C-X11-C-X2-CC). Within sub-family E one copy of *Anolis carolinensis OvoDE1* was located, three copies of *OvoDE2* and one copy of *OvoDE3* (Figure 1A, D, Table 4.4). One copy of each of the sub-family F members was identified, *OvoDF1*, *OvoDF2* and *OvoDF3* (Figure 4.1A, D, Table 4.4).



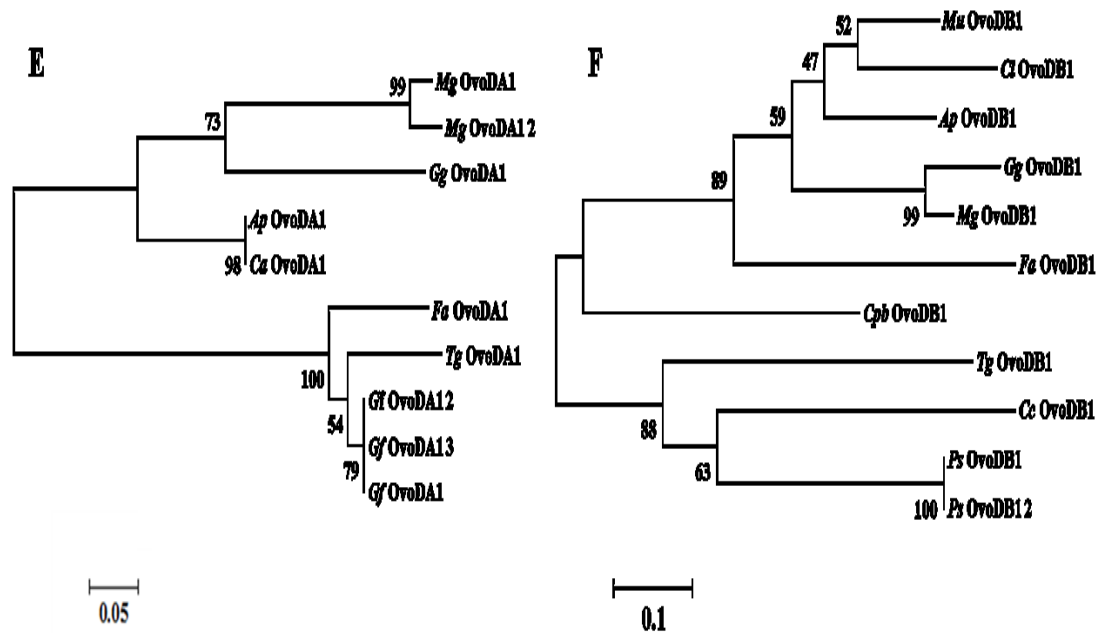


Figure 4.1: Evolutionary relationship of ovodefensin homologues. R 'hclust' was used to create a distance matrix and perform hierarchical clustering of all known ovodefensin members based on spacing between cysteine residues (A). This identifies six specific sub-families termed OvoDA-OvoDF. A phylogram indicating the evolutionary history of sub-families OvoDA, Ovodb, Ovodd and Ovode was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) from the amino acid sequence of a core region from the conserved glycine residue until the fourth cysteine (B). This suggested that the spacing with the cysteine motif of Ovodb evolved independently in avian and reptilian lineages and which are presented separately (C) and (D). Mature peptide sequences from sub-families OvoDA (E) and Ovodb (F) were analysed individually in order to compare the full length of the molecule.

For B, E and F the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The branch lengths are proportional to the evolutionary distances which were computed using the Poisson correction method (Zuckerandl and Pauling, 1965). The units are the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). A key for the abbreviations of the species Latin name can be found in Table 4.5 and the sequences the alignments are based on in Table 4.2.

Genome build and chromosome/scaffold locations for each ovodefensin are outlined in Tables 4.3 and 4.4.

Three homologs not previously classified as ovodefensins were also identified during this study. Shapiro et al (Shapiro et al., 2013) produced a rock pigeon reference genome from which a putative sub-family A member was identified. This had been named cygnin due to its homology with black swan cygnin and now propose the name *Columba livia OvoDA1* (Figure 4.1A, C, Table 4.4). From this genome a putative sub-family B member, named small basic protein was also located, the name *Columba livia OvoDB1* (Figure 4.1A, C, Table 4.4) is proposed for this ovodefensin homolog. In addition to this TEWP, a loggerhead turtle peptide was isolated from egg white and shown to be a defensin (Chattopadhyay et al., 2006), this peptide has the motif of a sub-family B ovodefensin and the name *Caretta caretta OvoDB1* is adopted (Figure 4.1A, C, Table 4.4).

4.4.2. 5'/3' RACE

5'/3' RACE using magnum RNA and primers as specified in 4.3.5 resulted in a PCR product with 100% identity to the published *Gallus gallus OvoDA1 (gallin)* sequence (ENSGALG00000028311.1), the 5' and 3' sequence of which was derived by prediction. Alignment with the chicken genome confirmed that *Gallus gallus OvoDA1* is encoded for by two exons.

4.4.3. Tissue expression

Gallus gallus OvoDB1 expression was restricted to magnum and isthmus as previously documented with *OvoDA1* (Gong et al., 2010); (Figure 10). No expression was observed in any other tissue tested for either *Gallus gallus OvoDA1* (Figure 4.2) or *OvoDB1* (Figure 4.3). In contrast to this both *Anas platyrhynchos OvoDA1* and *OvoDB1* expression was greatest in the shell gland, although some expression was observed in both the magnum and the isthmus (Figure 4.10). The combined expression of *Meleagris gallopavo OvoDA1* and *OvoDA1_2* and expression of *OvoDB1* was high across all oviduct tissues, the greatest expression for these turkey ovodefensins

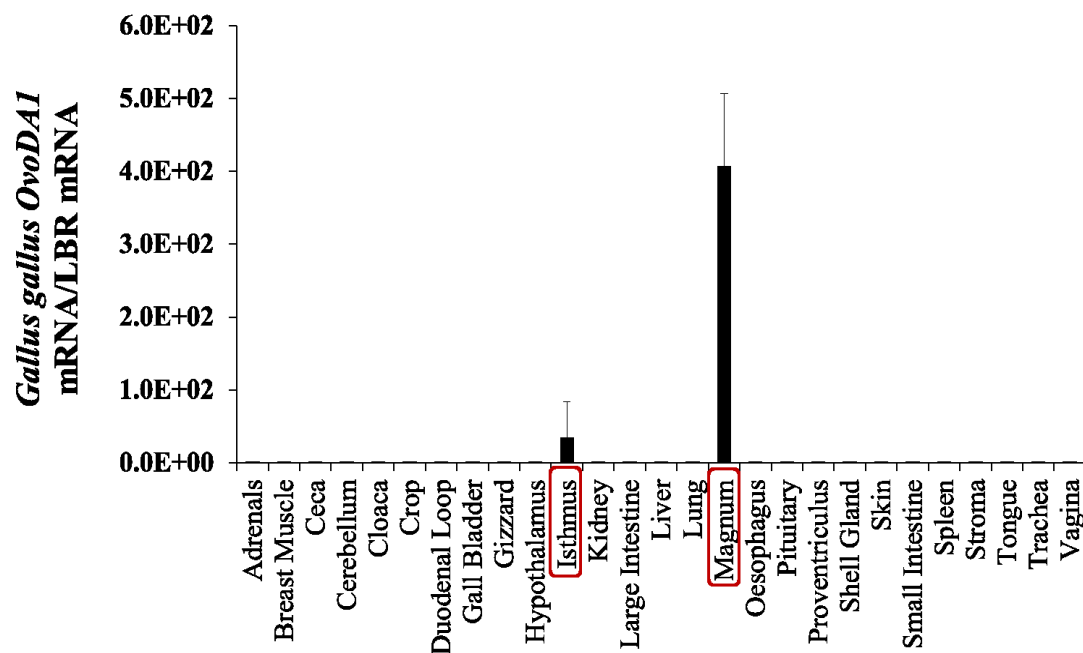


Figure 4.2: Expression of *Gallus gallus* OvoDA1 mRNA in a range of adult tissues measured by RT-QPCR (n=4, mean \pm sem). Expression was corrected for chicken LBR expression to normalise for any differences between tissues. Tissues showing expression are framed in red.

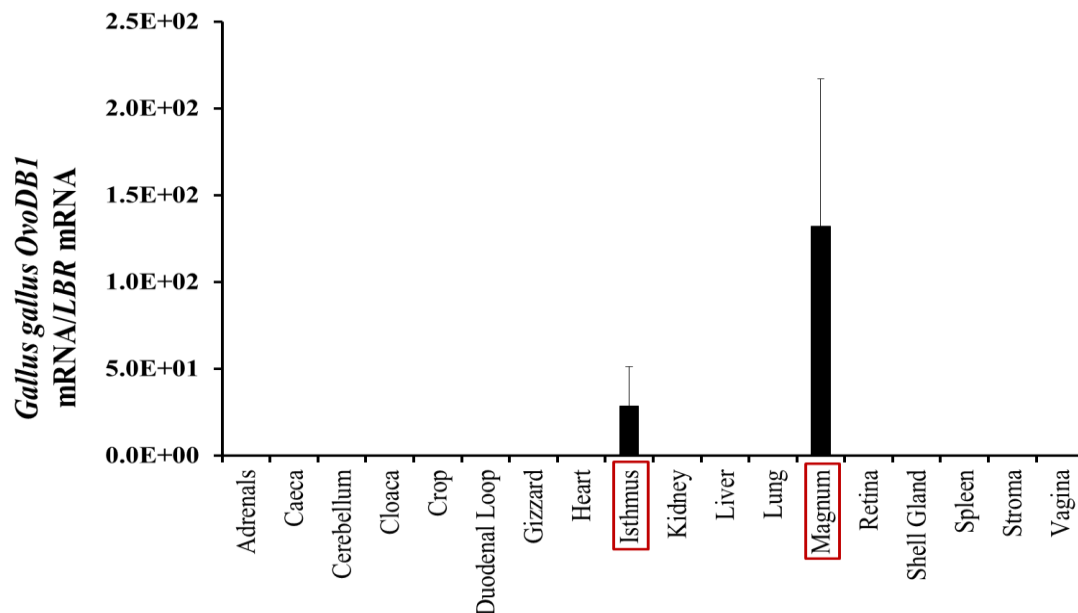


Figure 4.3: Expression of *Gallus gallus* OvoDB1 mRNA in a range of adult tissues measured by RT-QPCR (n=4, mean \pm sem). Expression was corrected for chicken LBR expression to normalise for any differences between tissues. Tissues showing expression are framed in red.

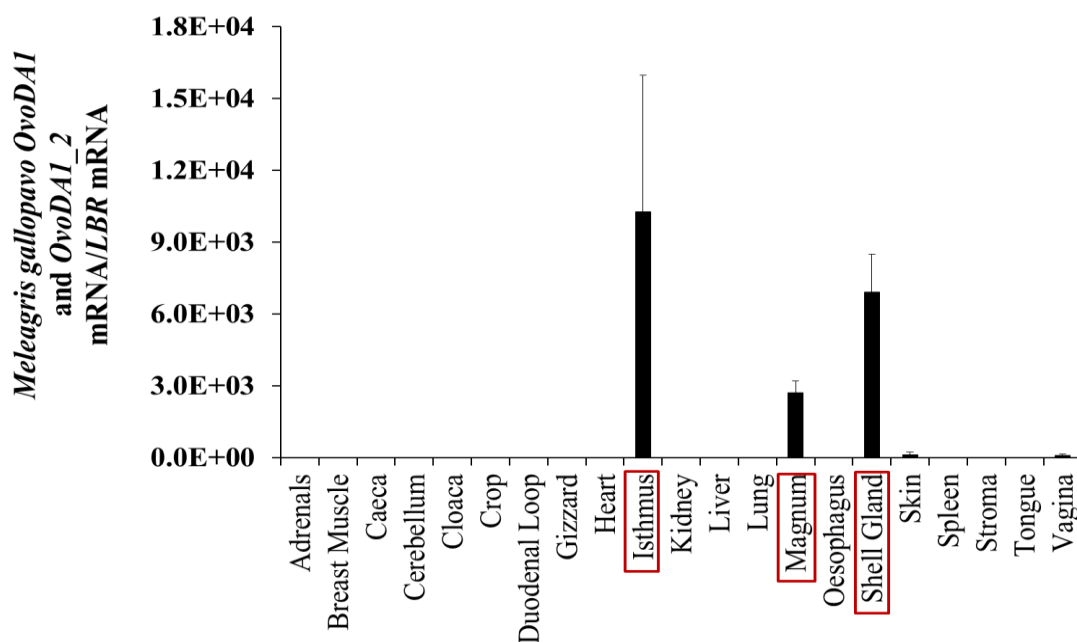


Figure 4.4: Combined expression of *Meleagris gallopavo* *OvoDA1* and *OvoDA1_2* mRNA in a range of adult tissues measured by RT-QPCR (n=4, mean \pm sem). Expression was corrected for turkey LBR expression to normalise for any differences between tissues. Tissues showing expression are framed in red.

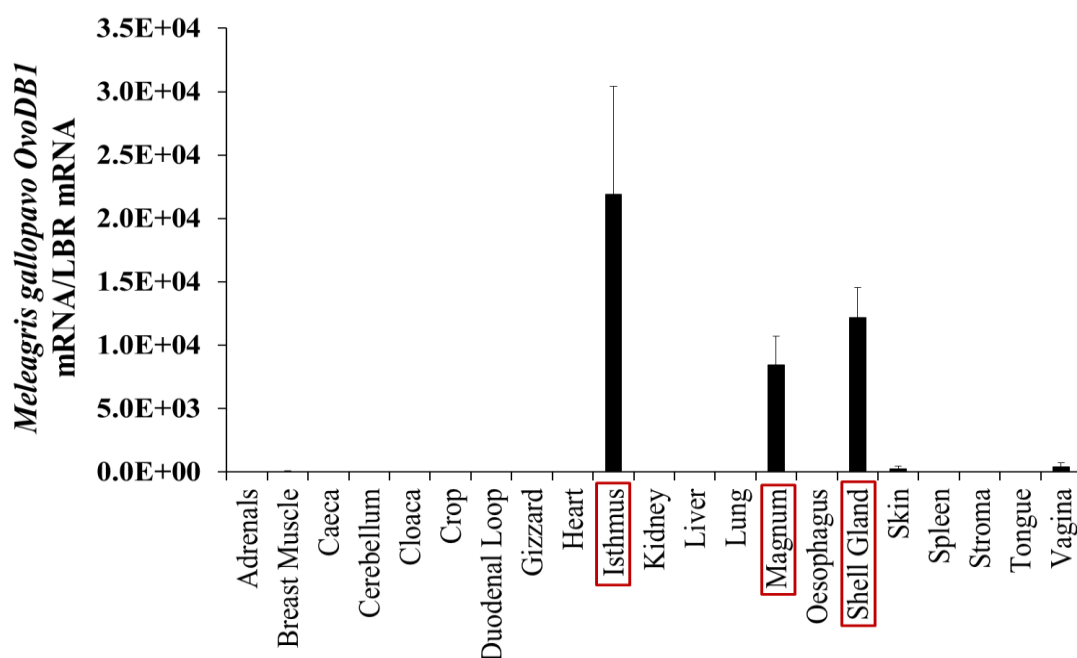


Figure 4.5: Expression of *Meleagris gallopavo* *OvoDB1* mRNA in a range of adult tissues measured by RT-QPCR (n=4, mean \pm sem). Expression was corrected for turkey LBR expression to normalise for any differences between tissues. Tissues showing expression are framed in red.

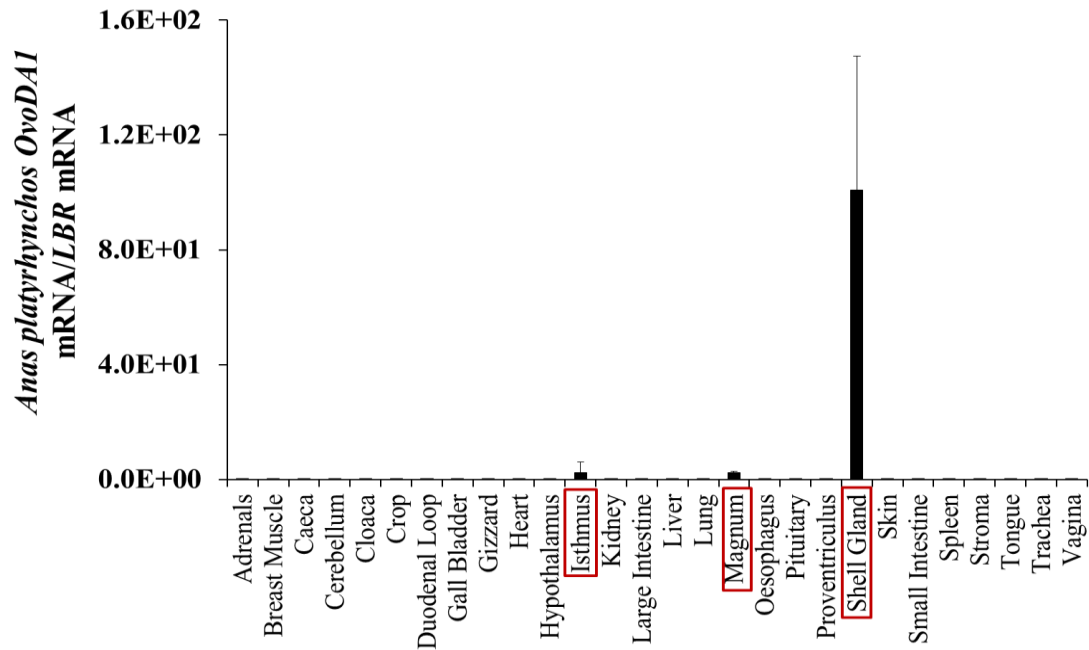


Figure 4.6: Expression of *Anas platyrhynchos* OvoDA1 mRNA in a range of adult tissues measured by RT-QPCR (n=3, mean \pm sem). Expression was corrected for turkey LBR expression to normalise for any differences between tissues. Tissues showing expression are framed in red.

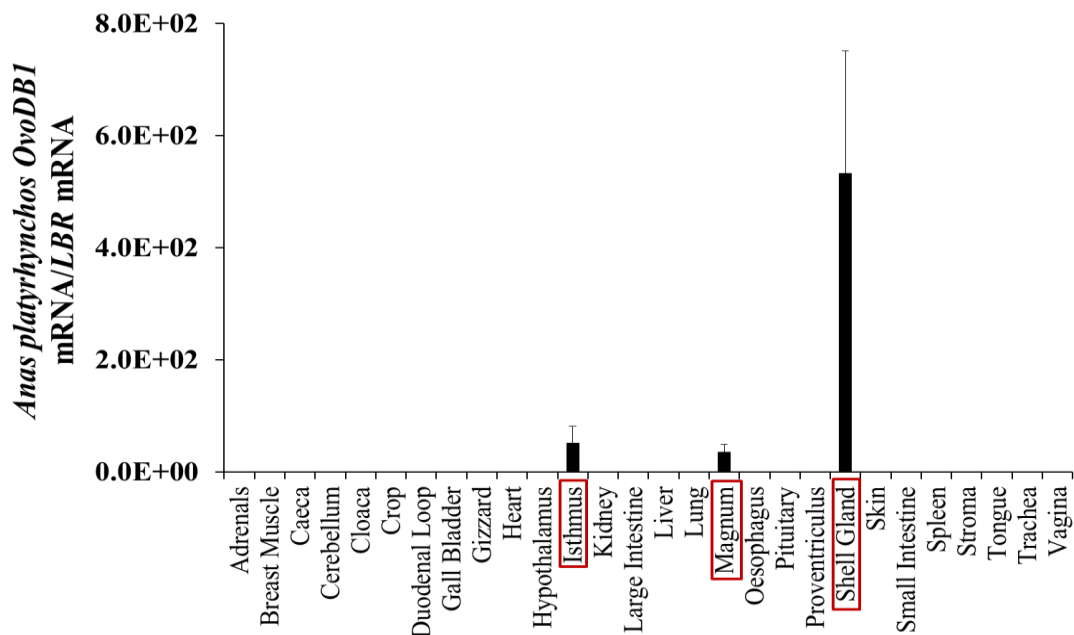


Figure 4.7: Expression of *Anas platyrhynchos* OvoDB1 mRNA in a range of adult tissues measured by RT-QPCR (n=3, mean \pm sem). Expression was corrected for duck LBR expression to normalise for any differences between tissues. Tissues showing expression are framed in red.

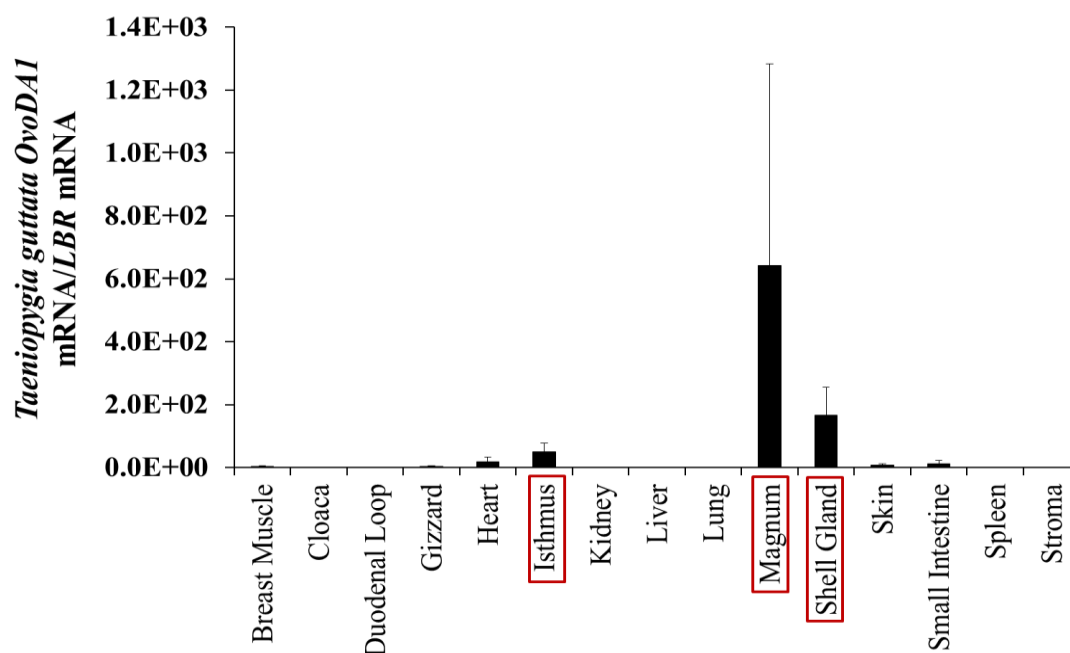


Figure 4.8: Expression of *Taeniopygia guttata* OvoDA1 mRNA in a range of adult tissues measured by RT-QPCR (n=5, mean \pm sem). Expression was corrected for zebra finch LBR expression to normalise for any differences between tissues. Tissues showing expression are framed in red.

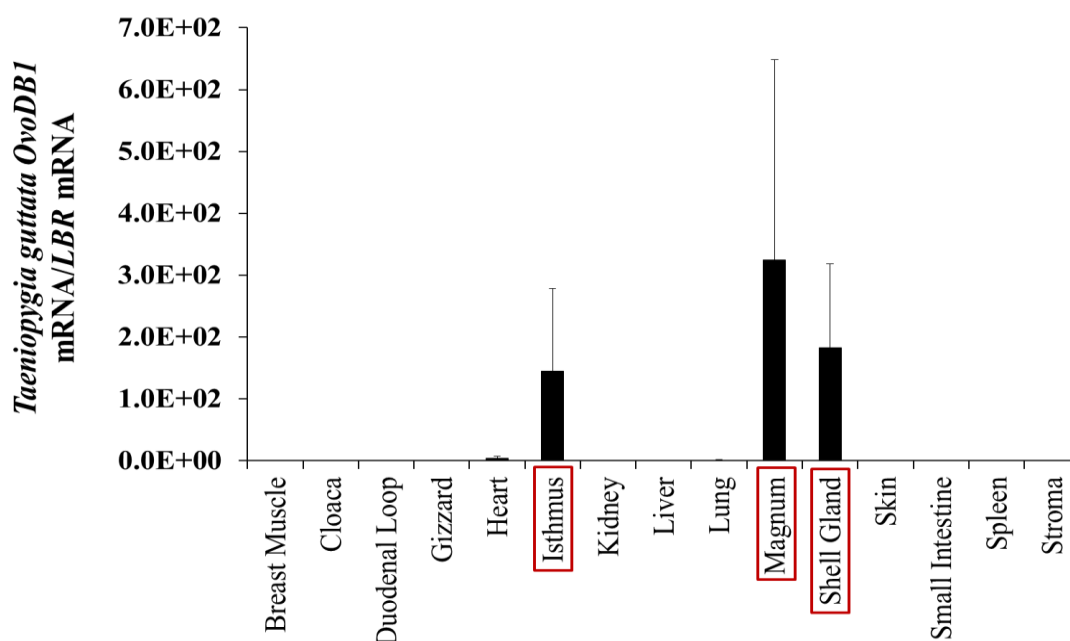


Figure 4.9: Expression of *Taeniopygia guttata* OvoDA1 mRNA in a range of adult tissues measured by RT-QPCR (n=5, mean \pm sem). Expression was corrected for zebra finch LBR expression to normalise for any differences between tissues. Tissues showing expression are framed in red.

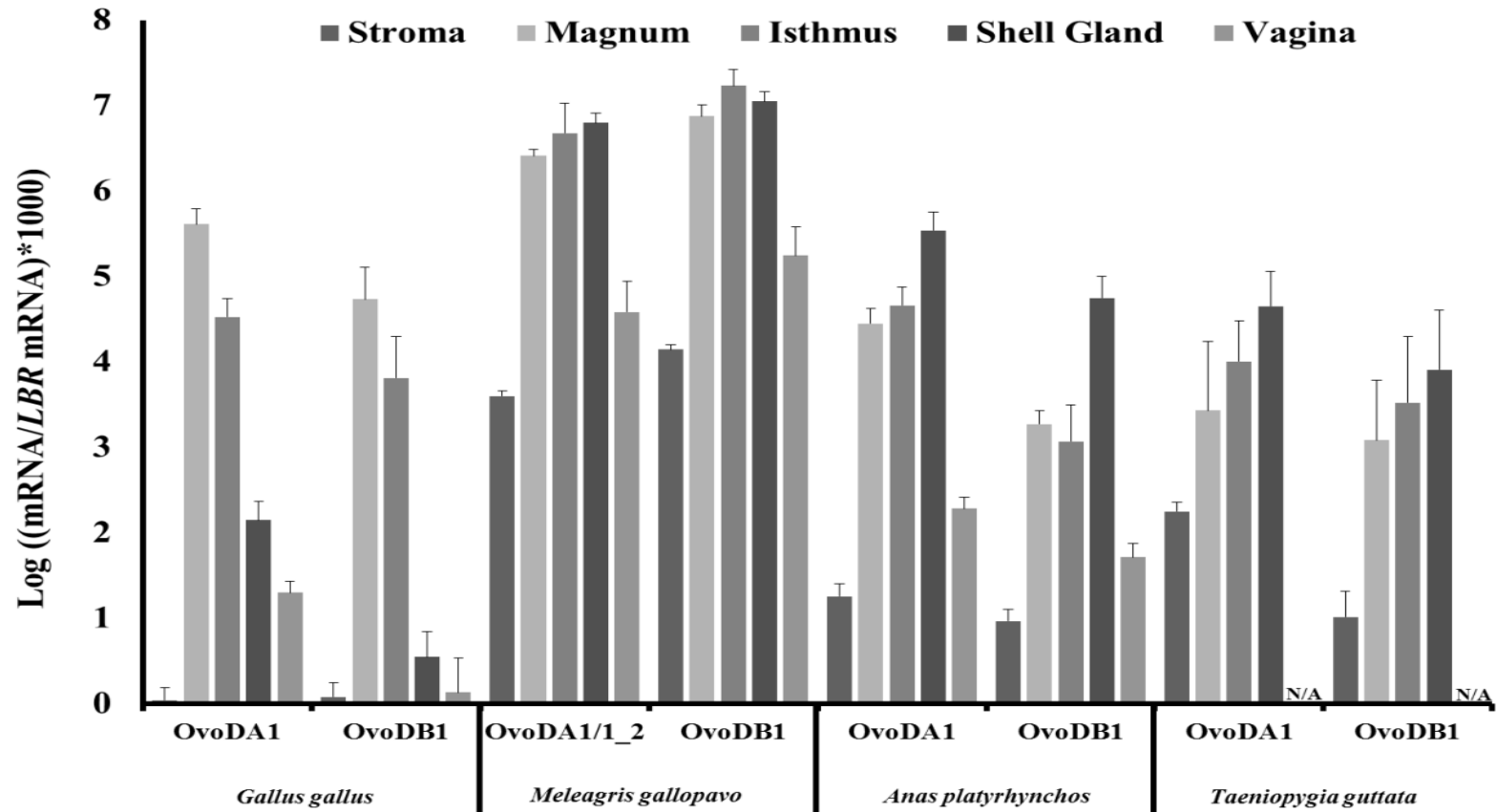


Figure 4.10: Expression of ovodefensin mRNA in a range of adult chicken (n=4, mean \pm sem), turkey (n=4, mean \pm sem), duck (n=3, mean \pm sem) and zebra finch (n=5, mean \pm sem) ovarian stroma and oviduct tissues measured by RT-QPCR (mean \pm sem). Expression was corrected for chicken, turkey, duck or zebra finch LBR expression to normalise for any differences between tissues. Note: to accommodate the large differences in expression the data are presented on the log scale.

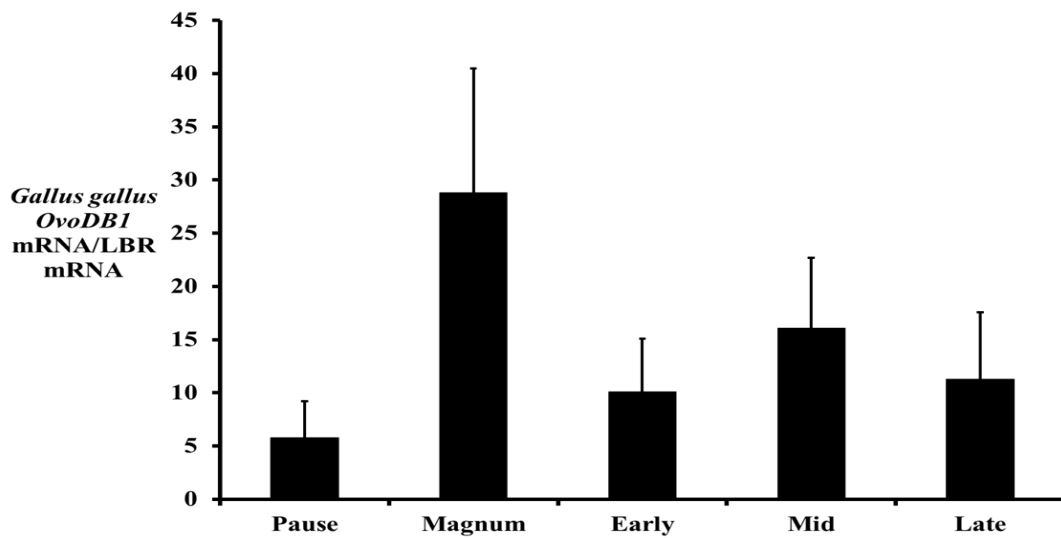


Figure 4.11: Expression of *Gallus gallus* *OvoDB1* mRNA in magnum tissue at different stages of egg formation measured by RT-QPCR (n=8, mean \pm sem). *Gallus gallus* *OvoDB1* expression was corrected for LBR expression. Pause represents a day when the hen did not ovulate so no egg is present. Magnum represents tissue taken when an egg is present in the magnum and early, mid and late describes the stage of shell formation in the shell gland and indicates the egg was not in the magnum when the sample was taken. ANOVA, $P=0.269$.

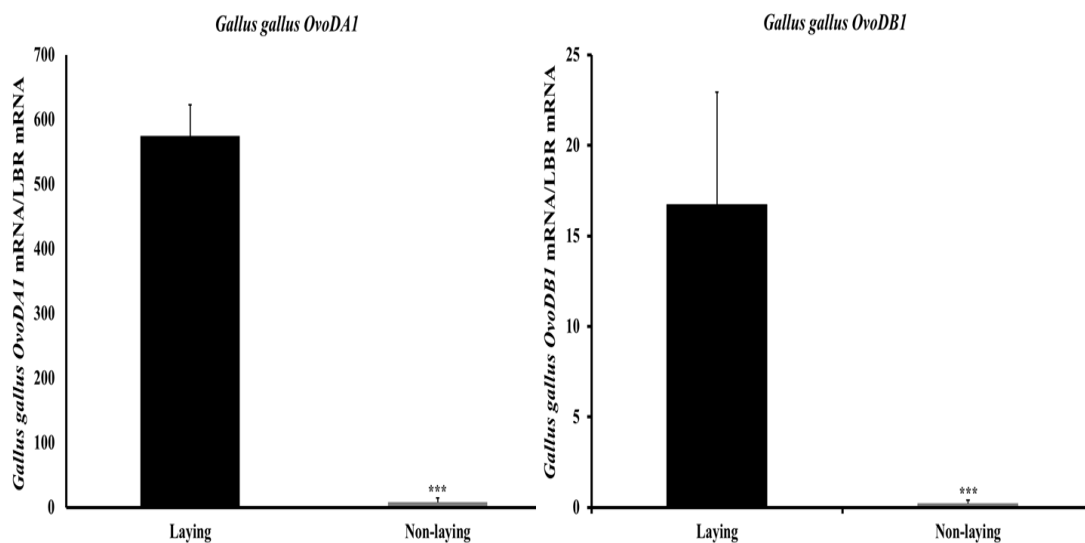


Figure 4.12: Expression of A) *Gallus gallus* *OvoDA1* and B) *Gallus gallus* *OvoDB1* in magnum tissue of laying (L) and out of lay (NL) birds measured by RT-QPCR (n=11, mean \pm sem). Non-laying hens were those where the oviduct had regressed due to the withdrawal of gonadotrophic support with the onset of incubation behaviour. Expression was corrected using LBR expression. Note the large difference in the Y-axis scale between A and B. Significance between laying state is indicated at $P<0.001$, (***).

was measured in the shell gland, magnum and isthmus (Figure 4.10). *Taeniopygia guttata* *OvoDA1* and *OvoDB1* expression was also restricted to the oviduct with the highest expression being observed in the shell gland for both genes (Figure 4.10). It should be noted that vagina tissue was not available for the zebra finch. For all the ovodefensin transcripts analysed, no expression was detected in tissues outside the reproductive tract (Figures 4.2-4.9).

There was no significant effect of the position of the egg in the oviduct or the lack of an egg in the oviduct on *Gallus gallus* *OvoDB1* expression in the magnum (Figure 4.11) (ANOVA, $P=0.269$). The level of *Gallus gallus* *OvoDA1* and *OvoDB1* expression in the magnum was higher ($P<0.001$) in birds in-lay than in those with an oviduct in the regressed state (Figure 4.12).

4.4.4. Immunohistochemistry

The chicken anti-OvoDB1 antiserum (108 CNK-1.3) produced positive staining in the tubular gland cells of the magnum (Figure 4.13A, C). The ciliated and non-ciliated cells lining the magnum region of the oviduct did not react to the primary antibody. No staining was observed in the isthmus, shell gland or caecum (data not shown). In contrast to this the zebra finch anti-OvoDB1 antiserum (113_KGE_2.1) produced positive staining in the tubular gland cells and surface epithelium of the magnum, isthmus and shell gland (Figure 4.14A, C, E). No convincing staining was observed in the breast muscle (Figure 4.14G).

4.5. Discussion

Seven ovodefensins had previously been identified within divergent avian species and it had been observed that two cysteine motifs exist (Gong et al., 2010); however it was clear that there may be further divisions in structure and therefore possibly function (Table 4.3). This study identified a further 25 ovodefensin members (Table 4.4) through genome analysis, and attributed a further 3 previously known sequences to the group expanding the ovodefensin family to include reptile species for the first time. It was first suggested by Gong *et al.* that the ovodefensins appeared to be a

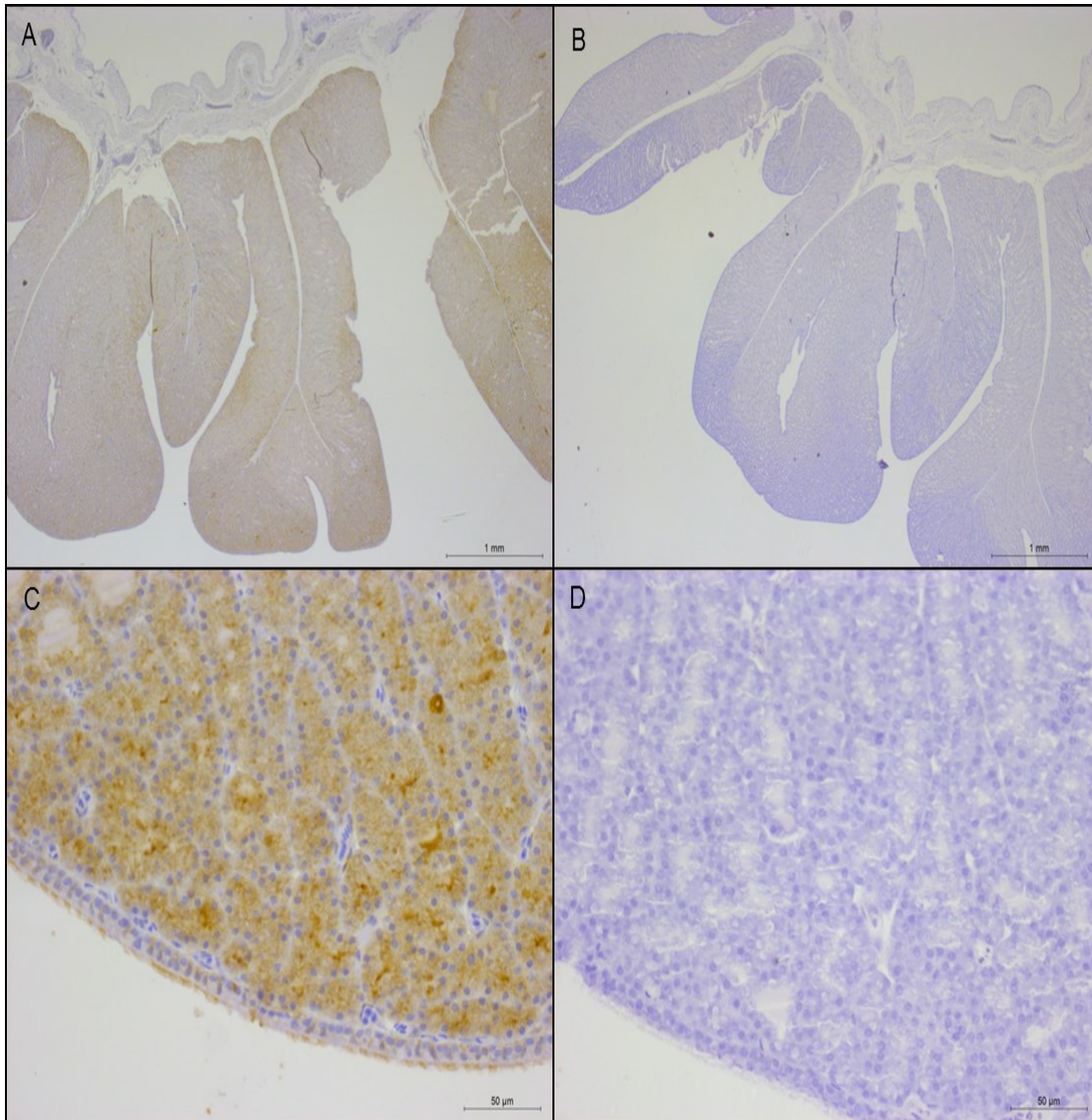


Figure 4.13: Only the tubular gland cells of the magnum (A and C) region of the oviduct convincingly stained positive with the chicken anti-OvoDB1 antisera (108 CNK-1.3) whereas the surface epithelial cells (ciliated and non-ciliated) stained negative (A and C). The corresponding negative controls for A and C are shown in B and D.

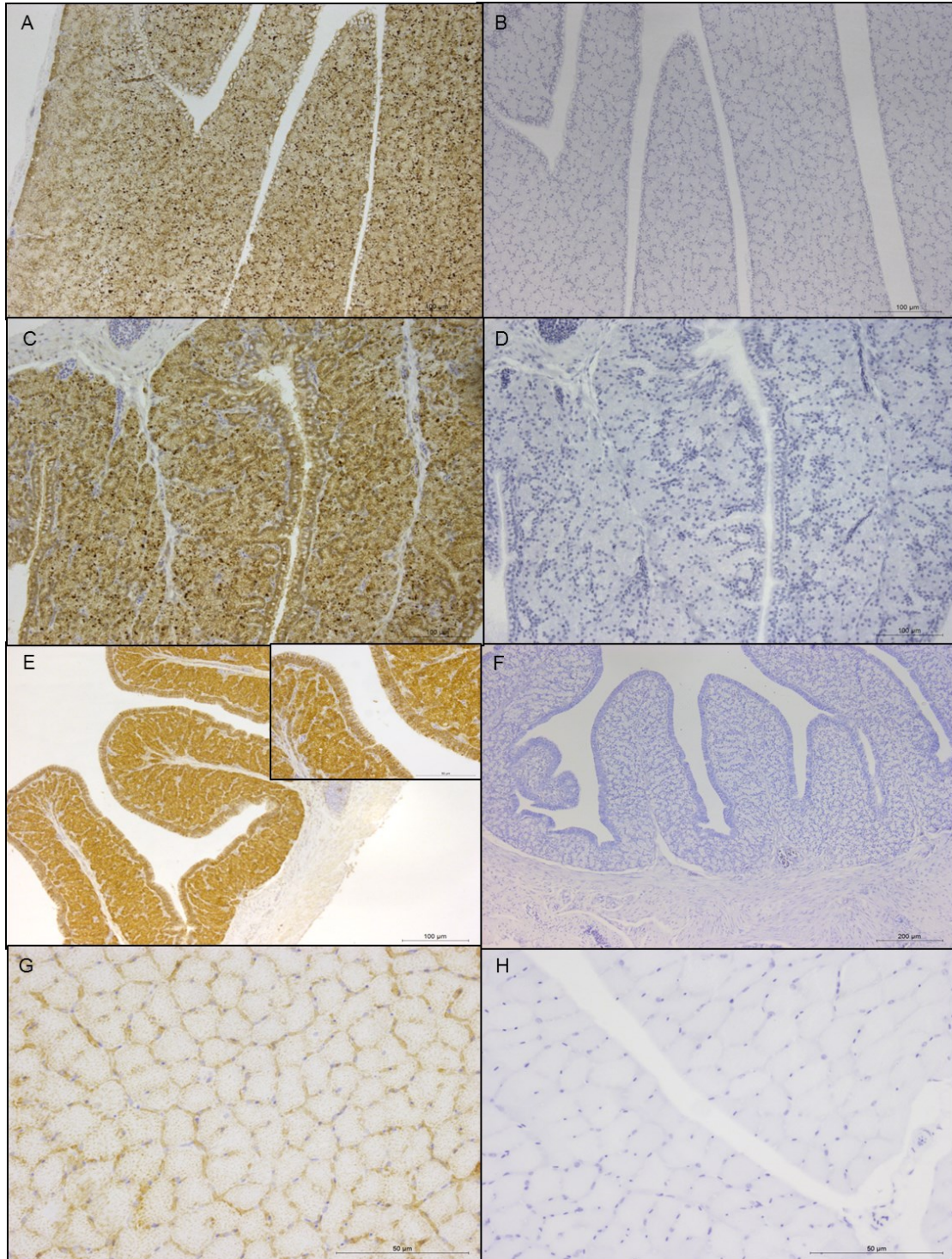


Figure 4.14: The tubular gland cells and surface epithelium of the magnum (A), isthmus (C) and shell gland (E) regions of the oviduct stained positive with zebra finch anti-OvoDB1 antisera (113_KGE_2.1). The corresponding negative controls are shown in image B, D, and F. Breast muscle (G and H) was not reactive to the primary antibody.

new family of defensin molecules, most likely belonging to the β -defensin group (Gong et al., 2010). However it was predicted that ovodefensins share the genomic organisation of mammalian β -defensins, being encoded for by two exons (Gong et al., 2010) rather than the four exons which encode for classical avian defensins (van Dijk et al., 2008). This study confirmed the exon arrangement of *Gallus gallus* *OvoDA1* by defining the transcription start site thus confirming the two exon predictions. Where available, predictions of the newly discovered avian and reptilian ovodefensins also support the 2 exon arrangement. In addition to an altered exon arrangement the ovodefensins differ from classical avian defensins in the spacing within the cysteine motif and therefore it is likely they form a new structural sub-family of defensins. This observation was supported by Hervé *et al* (2014) when the 3D structure and specific cysteine pairing was solved and it was demonstrated that *Gallus gallus* *OvoDA1* (gallin) contained an additional two stranded parallel β -sheet and no amino-terminal helix. Now phylogenetic analysis of both the spacing and sequence of the ovodefensins suggests that multiple sub-families may exist within the family (Figure 4.1A, C, D). These sub-families include four new ovodefensin-like motifs OvoDC (C-X3-C-X3-C-X13-C-X3-CC), OvoDD (C-X3-C-X3-C-X11-C-X3-CC), OvoDE (C-X6-C-X3-C-X11-C-X2-CC) and OvoDF (C-X6-C-X4-C-X11-C-X2-CC) revealing that the ovodefensin family is much more diverse than was first thought. All four new cysteine spacing motifs appear to be reptile specific (Figure 4.1A, D) whereas of the originally identified motifs OvoDA, (C-X5-C-X3-C-X11-C-X3-CC) was avian specific (Figure 4.1A, C) and OvoDB (C-X3-C-X3-C-X11-C-X4-C) contained both avian and reptilian counterparts (Figure 4.1A, C, D).

Phylogenetic analysis of spacing (Figure 4.1A) suggests that a common ancestor gave rise to two progenitor molecules which appear in turn to have independently evolved three cysteine motifs. However sequence analysis (Figure 4.1B) suggests that the OvoDB cysteine motif has in fact evolved separately within both avian and reptilian lineages spacing is an important feature of the ovodefensin molecules. In a sense this seems like a form of convergent evolution, albeit the available repertoire of spacing is relatively limited. In order to assess all the ovodefensin molecules

either spacing (Figure 4.1A) or a core region (Figure 4.1B) was used, however it is recognised that this may influence the outcome of the analysis depending on the evolutionary constraints of each selection. In particular, analysis based on the core region of the molecule (Figure 4.1B) produced some unexpected outliers, for example within the OvoDA cluster the turkey molecules appear closer to the duck and swan than the chicken. However when the full length peptides of the OvoDA sub-family are analysed separately (Figure 4.1E) a more classical species arrangement is observed. This demonstrates the complex nature of what appears to be the co-evolution of sequence and spacing and the need for both aspects to be studied in combination. Overall the evolution within birds, and now in this study another egg laying clade the reptiles, of a large repertoire of peptides that not only vary in sequence but the spacing between conserved cysteine residues suggests that the spacing between these cysteine residues may be critical for function. The region between the 1st and 2nd cysteine which varies from 3-6 amino acids in length for example has been demonstrated in *Gallus gallus* OvoDA1 to be important because of the basic residue in an otherwise hydrophobic region (Herve et al., 2014). It has been difficult to find examples in the literature of situations where the amino acid distance between conserved residues of a motif are changing or under selection, possibly because this is rare or because the methods of finding homologous genes relies heavily on the conservation of sequence, not pattern recognition. However approaches were developed to get round the problem in aligning large proteins where there were differences in spacing between conserved features important for protein secondary structure (Zhu et al., 1992) and these alignments appeared to have a better agreement with the accepted view of evolution than if this was not undertaken. In large proteins this has been used to demonstrate the evolution by insertion of new domains in molecules which have effects on structure and the authors conclude that using structure is likely to be more robust than sequence when molecules cannot be unambiguously aligned (Jiang and Blouin, 2007). In the case of the relatively small ovodefensins it also seems that this is the case.

RT-qPCR analysis on chicken, duck, turkey and zebra finch representatives of the ovodefensin family demonstrate that in all cases expression is restricted to the oviduct of the bird (Figures 4.2-4.9). However, interestingly, levels and patterns of expression within the oviduct vary between genes and species. As previously seen with *Gallus gallus OvoDA1* (Gong et al., 2010), *Gallus gallus OvoDB1* was expressed more highly in the magnum of the oviduct where the egg white is formed (Figure 4.10), however expression of *Gallus gallus OvoDA1* was more than 40 times that of *Gallus gallus OvoDB1* (Figure 4.12). In contrast to this both the duck and zebra finch ovodefensins were expressed most highly in the shell gland and the turkey ovodefensins had very high levels of expression in the magnum, isthmus and shell gland regions of the oviduct (Figure 4.10). Immunohistochemistry confirmed the expression of *Gallus gallus OvoDB1* (Figure 4.13) and *Taeniopygia guttata OvoDB1* (Figure 4.14) at a peptide level in specific regions of the oviduct. *Gallus gallus OvoDB1* peptide distribution was restricted to the tubular gland cells of the magnum suggesting it is secreted into the egg white as previously seen with *Gallus gallus OvoDA1*. In contrast *Taeniopygia guttata OvoDB1* was expressed in both the tubular gland cells and surface epithelium of the magnum, isthmus and shell gland suggesting this may play a greater role in local protection of the oviduct as well as the innate defence of the egg.

Gallus gallus OvoDB1 expression in the magnum of the oviduct did not differ significantly in relation to the position of the egg in the oviduct at the time of sampling (Figure 4.11); this was also the case for *Gallus gallus OvoDA1* (Gong et al., 2010). This expression profile is typical of egg proteins such as TENP (Whenham et al., 2014) or ovalbumin (Muramatsu et al., 1994). Both *Gallus gallus OvoDA1* and *OvoDB1* expression in the magnum was significantly higher when the hen was in-lay compared to when the oviduct was regressed (Figure 4.12), suggesting that these genes are under the control of gonadal steroids, therefore being specifically up-regulated during egg production when steroid levels are elevated (Lague et al., 1975). It is not possible to conclude if this is a direct effect of steroids

on the promoter or an indirect effect and indeed no classical oestrogen receptors were observed in the proximal promoter.

This chapter has demonstrated that ovodefensins are avian and reptilian specific members of the β -defensin family. Expression of avian members has been shown to be restricted to the oviduct and in the chicken are up-regulated in laying hens compared with hens with a regressed oviduct suggesting that gonadal steroids control expression. Although the chicken ovodefensins show the classic signature of an egg specific gene, the pattern across the range of species examined is that of an oviduct specific family. This coupled with the antimicrobial activity demonstrated in chapter 5 suggests that ovodefensins have specifically evolved for a role in egg defence as a component of the eggs innate chemical defence; however they may also contribute to maintaining sterility in the oviduct through local tissue activity. There is a large diversity within the ovodefensin family, with six motifs relating to spacing of the conserved cysteines discovered so far. This suggests that evolution is acting not only on amino acid sequence but also spacing of the molecule. This novel finding offers an additional avenue of investigation for the design of new antimicrobial compounds and makes the ovodefensins a promising family of molecules to take forward in this study. Their small size makes them less susceptible to proteolytic cleavage than TENP and their conservation across divergent avian and reptilian species is suggestive of an important protective function. With this in mind the next chapter will explore the antimicrobial function of this family and the prospects of these molecules for the modulation of gastrointestinal microflora.

Chapter 5

Antimicrobial function of the ovodefensin family

5.1. Introduction

Gallus gallus OvoDA1 (gallin), a chicken representative of the ovodefensin family was shown to be highly antibacterial against a laboratory-adapted strain of *E. coli* (Gong et al., 2010). It has since been suggested that its direct antimicrobial actions are limited to *E. coli* (Herve et al., 2014), however *Gallus gallus* OvoDA1 is so far the only member of this novel family to be studied. As it is hoped that host defence peptides such as ovodefensins may provide a new source of novel antimicrobials it is important to broaden our understanding of their capabilities including their activity against organisms of interest to the poultry industry such as Avian Pathogenic *E.coli*, *Salmonella* and *Campylobacter*. Many defensin molecules are salt sensitive (Ganz, 2003); and it is unclear if defensins are insensitive to pH. Insensitivity to factors like pH and salt concentration would assist their use as antimicrobial agents that aim to control gut health in monogastrics where the withdrawal of in-feed antimicrobials has been causing problems (Bedford, 2000). In order to overcome the cost associated with producing peptides in large quantities a fungal fermentation system has been used in this study (Chapter 2.14). It is important to understand if this production method affects activity, therefore fungal derived recombinant peptide will be evaluated against the traditionally tested synthetic peptide.

The purpose of this study was not to understand the role of the ovodefensins in the egg or oviduct but to determine whether or not they have the ability to modulate the gut microflora when administered orally through the feed and improve bird performance. This question cannot be answered just by studying their effects against isolated organisms *in vitro* even if specific conditions of the target organ such as pH are considered. However before progressing to the bird some promising evidence of activity is required in order to justify their use.

The limitations of testing naturally occurring antimicrobial peptides *in vitro* has long been under scrutiny and the potency of their activity is often directly affected by the conditions they are tested under (Schwab et al., 1999). Whether the peptide is tested under solid or liquid phase conditions or the absence/presence of salt, serum or

proteins in the test conditions have all been shown to affect activity (Schwab et al., 1999). The use of an *ex vivo* gut model allows the evaluation of promising candidates against a mixed community of microorganisms that directly resemble those conditions in which the peptide would need to work in terms of microbial microbiome structure, pH and metabolites. The model used in this study was developed by Alimetrix, Finland (Apajalahti et al., 2009) and evaluates the microbial and environmental changes to ileal and caecal environments after dosing with recombinant peptide.

5.2. Objectives

To test the hypothesis that ovodefensin activity is not restricted to *E.coli* by testing the antimicrobial capabilities of novel ovodefensin family members including the newly identified chicken sub-family B member against a range of microorganisms. This study also examined the effect of factors which may influence ovodefensin activity if used as a feed additive such as salt and pH. Finally an *ex vivo* gut model was used to investigate the hypothesis that ovodefensin peptides have the ability to modulate whole gut microbial communities.

5.3. Methods

5.3.1. Synthetic peptide production

Gallus gallus OvoDA1 (LVLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLPASWKWK), OvoDB1 (KRKGTCKGYCAPTCNKKDEWSFHQSCKKMYCCLLPLKKGK) and *Anas platyrhynchos* OvoDA1 (QVRKYCPKVGycSSKCSKADVWSLSSDCKFYCCLPPGWKGK) were synthesised (Almac Group, Gladsmuir, Scotland) (see 2.13). Freeze dried peptides were reconstituted in 10% dimethyl sulfoxide (DMSO). Synthetic peptide was used initially to screen peptides for activity in the *in vitro* assays.

5.3.2. Recombinant peptide production

Chemical synthesis of peptide is expensive and could not be used to produce sufficient yields for *ex vivo* and *in vivo* assays. Due to cost chemical synthesis would

also not be a commercially viable method of production for a feed additive and therefore peptide was produced in a recombinant system.

Recombinant *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1 were produced by Roal, Finland using a continuous fungal fermentation system. *Trichoderma* system expressing an empty vector was treated in the same way to act as a control in the *in vitro* and *ex vivo* evaluation (see 2.14).

Recombinant peptide was checked for antibacterial activity in the *in vitro* assay and used for the *ex vivo* assay and subsequently the *in vivo* trial detailed in Chapter 6.

5.3.3. Antimicrobial assay

A broth based antimicrobial assay (see 2.15) was used to determine the efficacy of *Gallus gallus* OvoDA1, OvoDB1 and *Anas platyrhynchos* OvoDA1. Final concentrations of 0.5, 1, 2.5, 5, 10, 25, 50 and 100 μ M were evaluated against *E. coli* K-12 strain DH5 α , avian pathogenic *E. coli* (APEC) O78:H9 strain χ 7122, *Salmonella enterica* serovars Enteritidis (SE125109) and Typhimurium (ST4/74). For *Staphylococcus aureus* (8325-4) final concentrations of 10, 25, 50, 100, 150 and 200 μ M were used. Results are expressed as a reduction in colony forming units per ml (CFU/ml) and where possible the effective dose 50 (ED50) was calculated using the DRC program in R (<http://www.bioassay.dk/>) (see 2.15.5).

Neat purified recombinant *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1 and control supernatant (see 5.3.2) were also evaluated in the antimicrobial assay before testing in the *ex vivo* model.

Gallus gallus OvoDA1 was also evaluated against *Campylobacter jejuni* 11168 which is known to possess genes which confer resistance to cationic antimicrobial peptides as well as a mutant of this strain 11168H which lacks these genes (see 2.15.1 for details). The same broth based assay as described previously was used with specific media and incubation conditions (see 2.15.1).

5.3.3.1. pH sensitivity

The antimicrobial assay as outlined above (5.3.2) was adapted to test the effect of pH on the efficacy of *Gallus gallus* OvoDA1 against *E. coli* DH5 α . The assay was carried out as before but using PBS at pH 6.4, 7.4 and 8.4; details can be found in appendix 1. *Gallus gallus* OvoDA1 was tested at final concentrations of 1.9, 5.6, 16.7 and 50 μ M.

5.3.3.2. Salt sensitivity

The antimicrobial assay (5.3.2) was adapted to test the effect of salt sensitivity on *Gallus gallus* OvoDA1 efficacy. PBS with NaCl concentrations of 50, 100 and 150 mM, pH 6.4 were used; see appendix 1 for details.

5.3.3.3. Bacterial metabolic state

Some traditional antimicrobials are only able to kill replicating bacteria. To determine if metabolic state has an effect on *Gallus gallus* OvoDA1 activity it was assessed against Avian Pathogenic *E. coli* in both a metabolically active and static state at 10 μ M (see 2.15.4). Ampicillin was used as positive control in these experiments as it is known to interfere with cell wall synthesis and therefore requires bacterial cells to be metabolically active.

5.3.4. Viral plaque assay

The antiviral activity of synthetic *Gallus gallus* OvoDA1, OvoDB1 and recombinant *Taeniopygia guttata* OvoDB1 was evaluated against a H1N1 Influenza A virus (A/WSN/33) using a eukaryotic MDCK cell monolayer (see 2.16). *Gallus gallus* OvoDA1 and OvoDB1 were tested at 0.001, 0.01, 0.1, 1, 10 and 100 μ M and recombinant *Taeniopygia guttata* OvoDB1 was used neat and diluted 1:2. A known antiviral peptide (FluPep) (Nicol et al., 2012) was used as a positive control at 0.1, 1 and 10 μ g (see 2.16). Results are expressed as a % reduction in viral plaques per well.

5.3.5. Macrophage infection assay

The ability of *Gallus gallus* OvoDA1 at 1 and 10 μ M to directly activate a macrophage-like cell line (HD11) was evaluated using *Salmonella*-gentamicin assay detailed in 2.17. The assay measures the net total number of *Salmonella* phagocytised by the macrophages as well as the intracellular *Salmonella* replication of those phagocytised; extracellular *Salmonella* is eliminated from the final count using gentamicin. Results are expressed as a % reduction in *Salmonella* colony forming units per well.

5.3.6. *Ex vivo* gut model

The method is adapted for poultry from work published on pigs (Apajalahti et al., 2009). The *ex vivo* model and analysis of environmental parameters including pH, gas production and short chain fatty acid (SCFA) profile was carried out by Alimetrics, Finland, I assisted with the DNA extraction/purification and RT-QPCR analysis of the microbial community.

More information on this method can be found in chapter 2 (2.18). Briefly, ileal or caecal contents from 30 4-week old chickens were pooled and the conditions of incubation were adjusted to mimic chicken ileal and caecal conditions as accurately as possible. For the simulations, recombinant *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1 were tested at final concentrations of 50, 100 and 200 μ M, control supernatant was tested at a dose equivalent to 100 μ M of recombinant peptide. A negative control containing no additional supplement was included for comparison.

After 10 hours, the volume of gas released from the vessels was recorded. After opening the simulation vessel, the pH of the fermentation medium was measured, this measurement was done immediately after the gas measurement to avoid pH shifts caused by the escape of CO₂ from the medium. The medium was then sampled for the analysis of short chain fatty acids (SCFA) and bacterial density. The acids measured were acetic, propionic, butyric, iso-butyric, 2-methyl-butyric, valeric, and

lactic acid. Total bacterial numbers and specific genus or species were enumerated using RT-qPCR.

Treatments were compared to the negative control using ANOVA to identify significant differences and least significant difference was then used to test between means when significance of $P < 0.05$ was observed in the ANOVA. Bacterial counts were transformed (\log_{10}) before analysis to normalise the data. To demonstrate clearly if a significant difference was peptide specific or a result of background fungal components the data from LSD is also shown separately to compare the negative control, supernatant control and 100 μM treatments as these, at least theoretically contain the same amount weight/weight of background fungal components.

5.4. Results

5.4.1. Antimicrobial activity

5.4.1.1. Synthetic peptide

Gallus gallus OvoDA1 (gallin) peptide achieved a relatively large effect on *E. coli* DH5 α with around a 98% reduction in CFU/ml at 100 μM (Figure 5.1A, Table 5.1), and is comparable to the results reported previously with *E. coli* BL21 (Gong et al., 2010). *Gallus gallus* OvoDA1 achieved around a 40% reduction in viability of avian pathogenic *E. coli* O78:H9 strain χ 7122 when used at 50 or 100 μM (Figure 5.1A, Table 5.1) and a >90% reduction in viability against *S. aureus* 8325-4 a Gram-positive organism, when used at 100 or 200 μM (Figure 5.1B, Table 5.1). No reduction was observed against either of the *Salmonella* strains used in this study (Table 5.1). A 35% reduction in viability of *E. coli* DH5 α was achieved with 100 μM *Gallus gallus* OvoDB1 (Figure 5.2), and a very small reduction was observed in APEC numbers (~3%) (Table 5.1). No reduction was observed with *Gallus gallus* OvoDB1 against *S. aureus* or the *Salmonella* strains tested (Table 5.1). A duck representative of the ovodefensin family, *Anas platyrhynchos* OvoDA1 (dBPS₂) showed around an 80% reduction in viability of *E. coli* DH5 α (Figure 5.3)

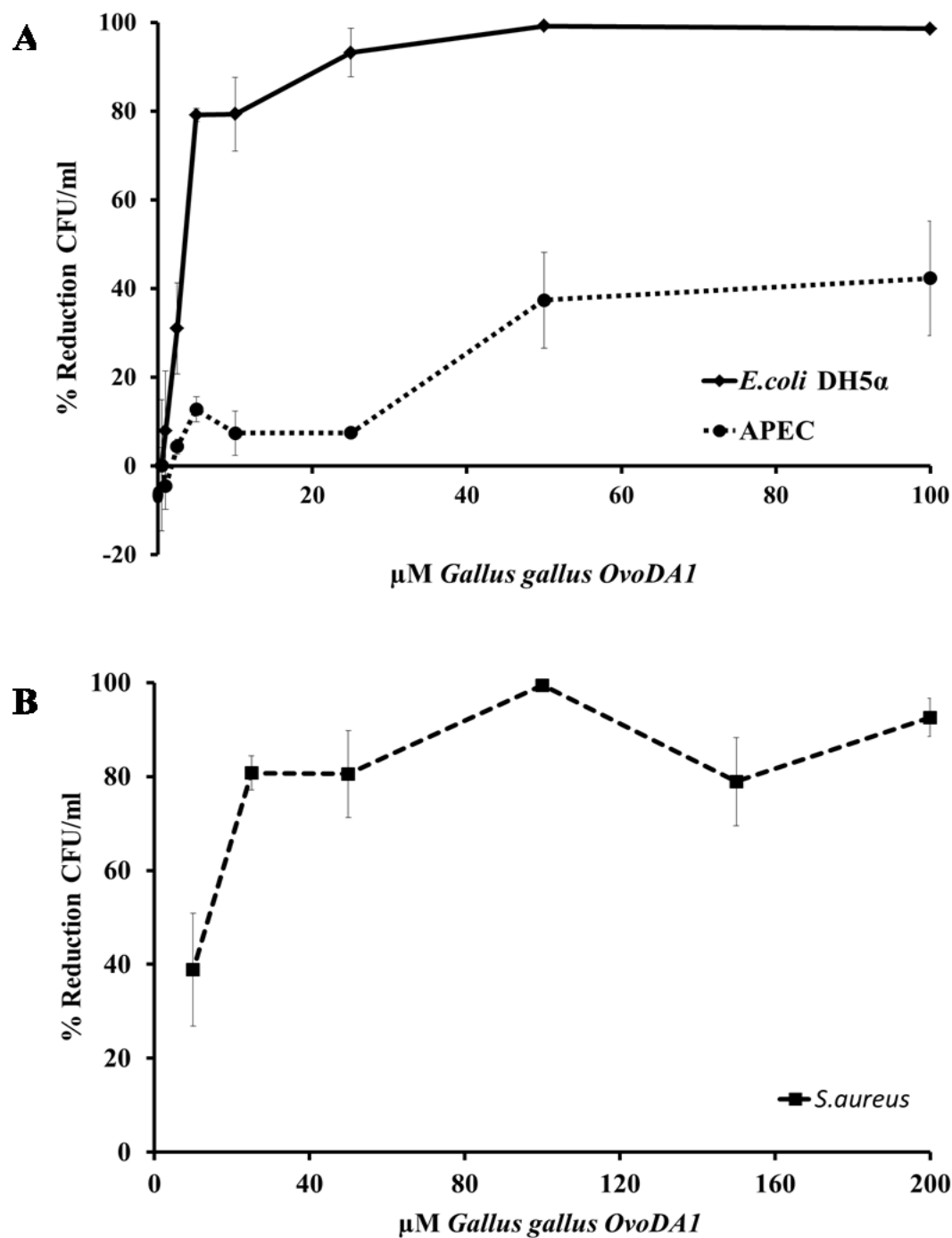


Figure 5.1: *Gallus gallus* OvoDA1 was incubated for 3 hours at 37°C with *E.coli* DH5α, Avian Pathogenic *E.coli* χ 7122 at 0.5-100 μ M (A) or *S.aureus* 8325-4 at 10-200 μ M (B) in PBS and the number of surviving bacteria were counted ($n=3$, mean \pm sem). Results are represented as a reduction in CFU/ml when compared to a 10% DMSO control.

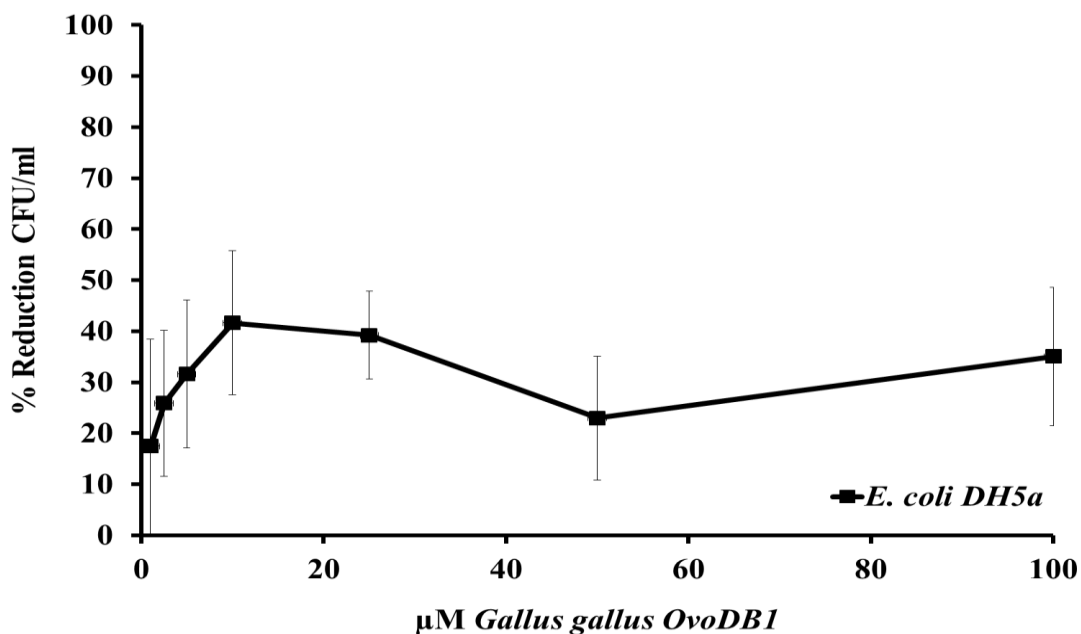


Figure 5.2: *Gallus gallus* OvoDB1 was incubated for 3 hours at 37°C with *E.coli* DH5α in PBS and the number of surviving bacteria were counted (n=3, mean ± sem). Results are represented as a reduction in CFU/ml when compared to a 10% DMSO control.

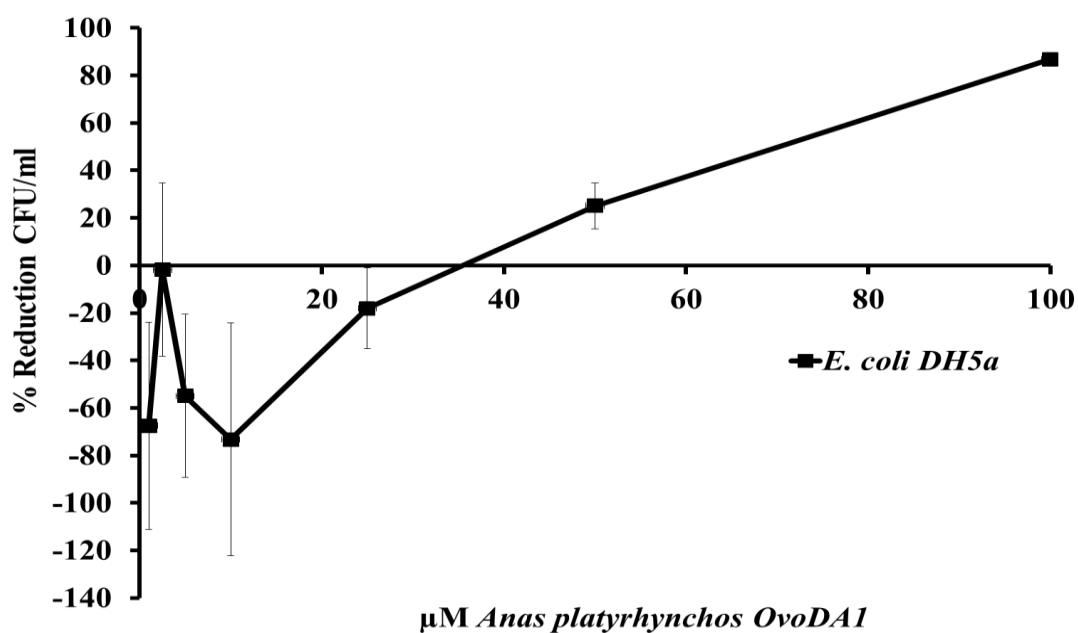


Figure 5.3: *Anas platyrhynchos* OvoDA1 was incubated for 3 hours at 37°C with *E.coli* DH5α in PBS and the number of surviving bacteria were counted (n=3, mean ± sem). Results are represented as a reduction in CFU/ml when compared to a 10% DMSO control.

<u>Synthetic</u>						
	<i>Gallus gallus</i> OvoDA1		<i>Gallus gallus</i> OvoDB1		<i>Anas platyrhynchos</i> OvoDA1	
	% reduction ¹	ED50 ²	% reduction ¹	ED50 ²	% reduction ¹	ED50 ²
DH5α	99	3	35	-	87	53
APEC	42	-	3	-	-	-
<i>S. aureus</i>	99	16	-	-	-	-
<i>S. enteritidis</i>	-	-	-	-	-	-
<i>S. typhimurium</i>	-	-	-	-	-	-
<i>C. jejuni</i> (WT)	10	-	X	X	X	X
<i>C. jejuni</i> (mut)	41	-	X	X	X	X
<u>Recombinant</u>						
	<i>Gallus gallus</i> OvoDA1		<i>Taeniopygia guttata</i> OvoDB1			
	% reduction ¹	ED50 ²	% reduction ¹	ED50 ²		
DH5α	83	-	98	-		

Table 5.1 – Summary of antimicrobial activity of synthetic *Gallus gallus* OvoDA1, *Gallus gallus* OvoDB1 and *Anas platyrhynchos* OvoDA1 and recombinant *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1. Percent reduction in CFU/ml at 100 μM is shown for each peptide when activity is observed¹. The ED50 value of synthetic peptides was calculated where a fall in viability greater than 50% was observed². ‘X’ is used when an organism was not tested against that peptide. Bacterial strains used were *E. coli* K-12 (DH5α), avian pathogenic *E. coli* O78:H9 (χ7122), *Salmonella enterica* serovars Enteritidis (SE125109) and Typhimurium (ST4/74), *Staphylococcus aureus* (8325-4) and *Campylobacter jejuni* 11168 wild type (WT) and 11168H mutant (mut).

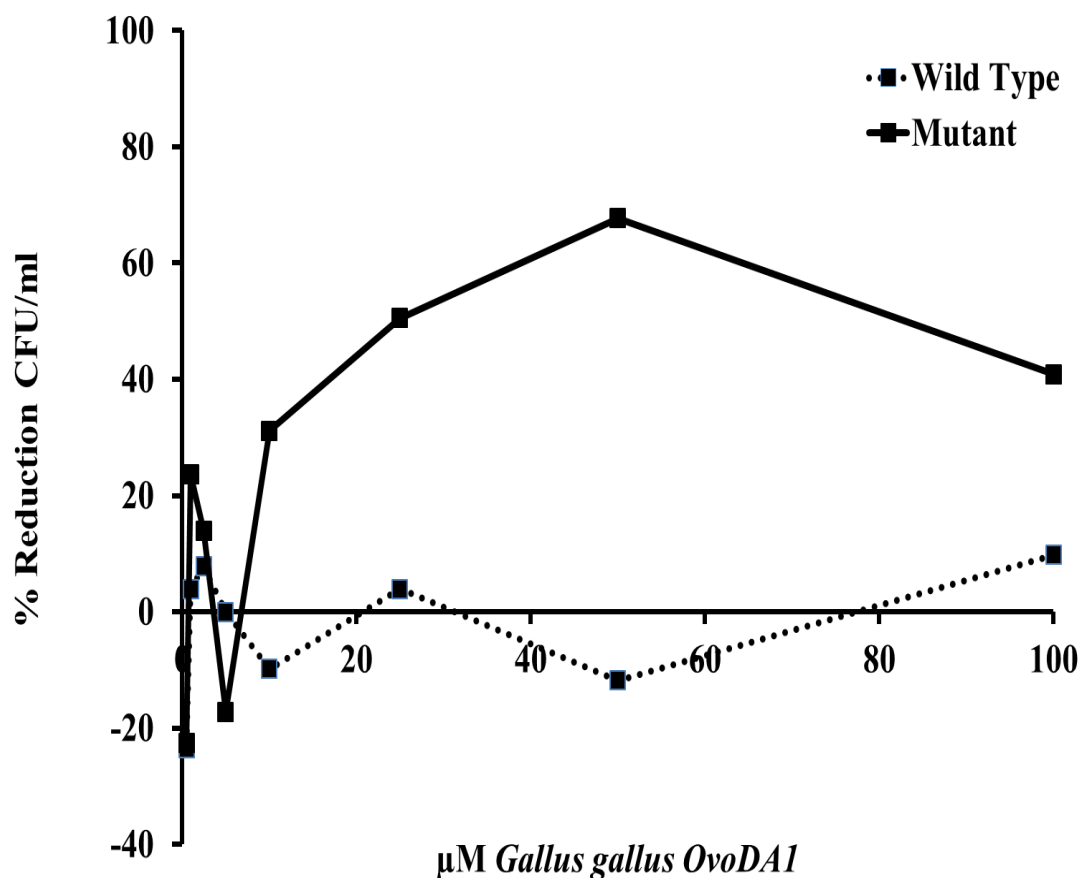


Figure 5.4: *Gallus gallus* OvoDA1 was incubated for 3 hours at 37°C with *Campylobacter jejuni* 11168 (wild type) or *Campylobacter jejuni* 11168H (mutant) at 0.5-100 µM in PBS and the number of surviving bacteria were counted (n=1). Results are represented as a reduction in CFU/ml when compared to a 10% DMSO control. Note: due to difficulty in culturing these strains the experiment was not repeated.

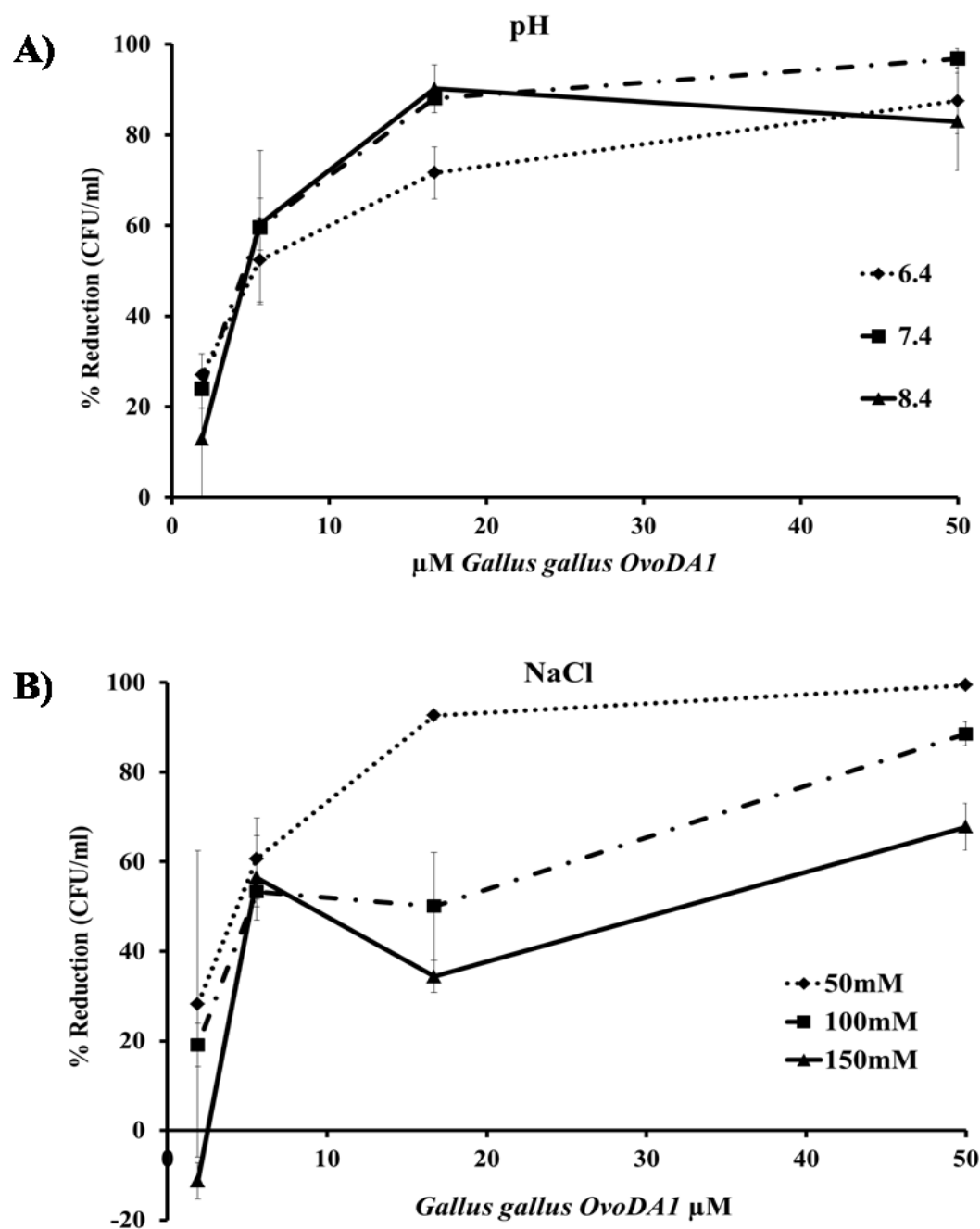


Figure 5.5: *Gallus gallus* OvoDB1 was incubated for 3 hours at 37°C with *E.coli* DH5 α in PBS at a range of pH (A) and NaCl concentration (B) and the number of surviving bacteria were counted (n=3, mean \pm sem). Results are represented as a reduction in CFU/ml when compared to a 10% DMSO control.

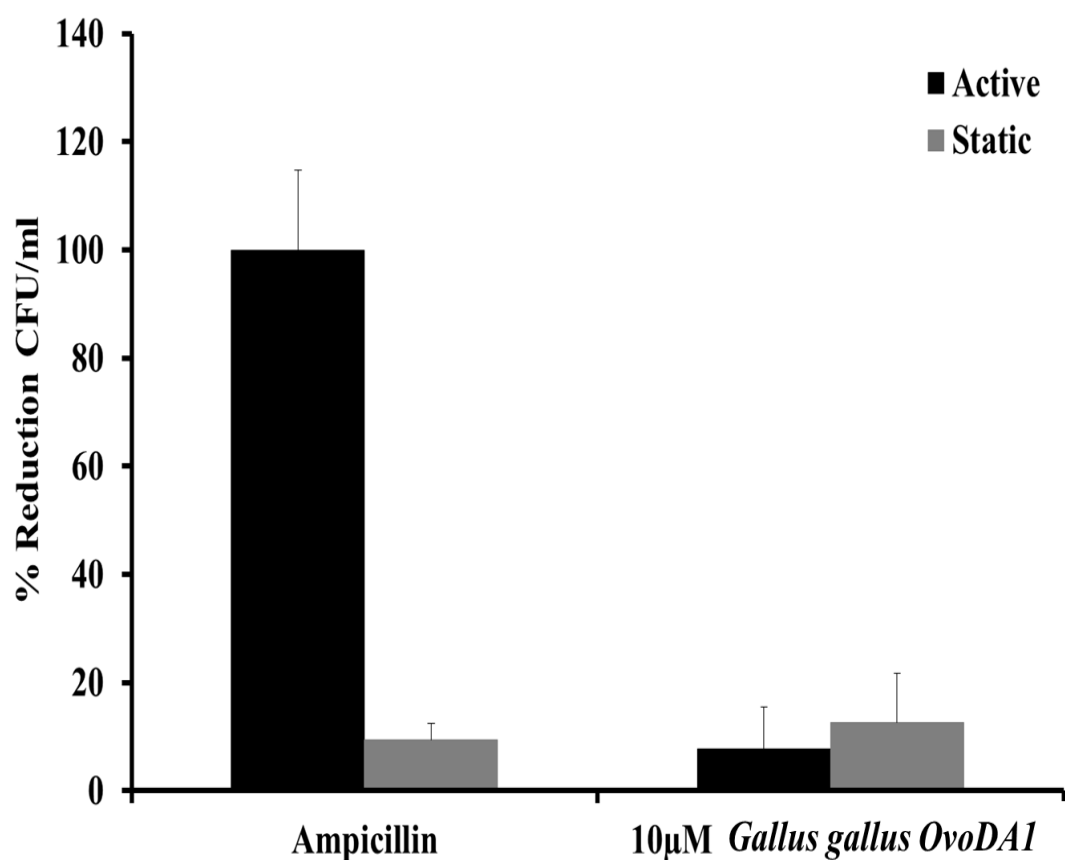


Figure 5.6: *Gallus gallus* OvoDA1 was incubated for 3 hours at 37°C with metabolically active Avian Pathogenic *E. coli* χ 7122 or metabolically static Avian Pathogenic *E. coli* χ 7122 at 10 µM and the number of surviving bacteria were counted (n=3, mean \pm sem). Results are represented as a reduction in CFU/ml when compared to a 10% DMSO control.

but no convincing activity was seen against any of the other bacterial strains tested (Table 5.1).

Gallus gallus OvoDA1 achieved a slight reduction in wild type *Campylobacter jejuni* CFU/ml (9.8%) at 100 μ M (Figure 5.4); however at the same concentration a 41% reduction was observed in the mutant strain which lacks known cationic peptide resistance genes (Figure 5.4).

Gallus gallus OvoDA1 potency as measured by ED50 did not differ significantly due to pH ($P=0.42$), the ED50 of *Gallus gallus* OvoDA1 at pH 6.4, 7.4 and 8.4 was 7.38 μ M, 3.57 μ M and 3.67 μ M respectively (Figure 5.5A). The potency of *Gallus gallus* OvoDA1 at ED50 level was also not affected by salt concentration ($P=0.49$), the ED50 of *Gallus gallus* OvoDA1 at 50, 100 and 150 mM NaCl was 3.55 μ M, 3.88 μ M, and 3.27 μ M respectively (Figure 5.5B). However at higher concentrations of *Gallus gallus* OvoDA1 antibacterial activity appeared to be diminished in a manner sensitive to the salt concentration (Figure 5.5B).

The metabolic state of APEC did not affect the antibacterial activity of *Gallus gallus* OvoDA1 with a 10% reduction being observed in both the metabolically active and static bacteria (Figure 5.6). The difference in metabolic states can be observed clearly by the antibacterial effect of ampicillin which resulted in nearly a 100% reduction in CFU/ml when the cells were metabolically active and 10% when the bacteria was in a static state (Figure 5.6).

5.4.1.2. Recombinant peptide

Neat recombinant *Gallus gallus* OvoDA1 achieved an 83% reduction in *E. coli* DH5 α , *Taeniopygia guttata* OvoDB1 also showed potent antimicrobial activity with a 98% reduction in CFU/ml (Table 5.1). No antibacterial activity was detected for the supernatant control.

5.4.2. Anti-viral activity

No anti-viral activity was observed for *Gallus gallus* OvoDA1, OvoDB1 or recombinant *Taeniopygia guttata* OvoDB1 against Influenza A/WSN/33 in the assay used. On average *Gallus gallus* OvoDA1 resulted in a 6% increase in viral plaques when compared to the control. *Gallus gallus* OvoDB1 gave a 5% increase and recombinant *Taeniopygia guttata* an 8% increase; however these results were not statistically significant due to variability in plaque number. No toxicity was observed to the eukaryotic cell line used for either of the synthetic peptides or recombinant form. FluPep (see 2.16) achieved around an 88% reduction at 100 µg.

5.4.3. Macrophage infection assay

Administration of *Gallus gallus* OvoDA1 to HD11 cells resulted in a 4% increase in extracellular *Salmonella* in the first assay. As this result did not indicate an ability to activate macrophage activity the experiment was not repeated due to the large quantity of peptide required.

5.4.4. Ex vivo gut microbiome modulation

5.4.4.1. Ileum

A significant effect was observed on the amount of gas in the *ex vivo* ileum (ANOVA, $P < 0.001$) (Figure 5.7A). However, although an increase was recorded when treated with *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1, supernatant alone also resulted in an increase in gas (Figure 5.7B). Ileum pH was significantly reduced in all treatments including the supernatant control (ANOVA, $P < 0.001$) (Figure 5.7C and 5.7D). Acetic acid concentration (mM) significantly increased in all treatments including the supernatant control (ANOVA, $P < 0.001$) (Figure 5.8A and 5.8B), however the relative amount of acetic acid (%) 100 µM *Gallus gallus* OvoDA1 resulted in a significant reduction whereas the supernatant resulted in a significant increase when compared with the negative control (Figure 5.8C and 5.8D). A significant effect on lactic acid (mM) was observed (ANOVA, $P < 0.001$). 100 µM *Gallus gallus* OvoDA1 resulted in a significant increase in lactic acid compared with both the control and the supernatant (Figure 5.9A and 5.9B).

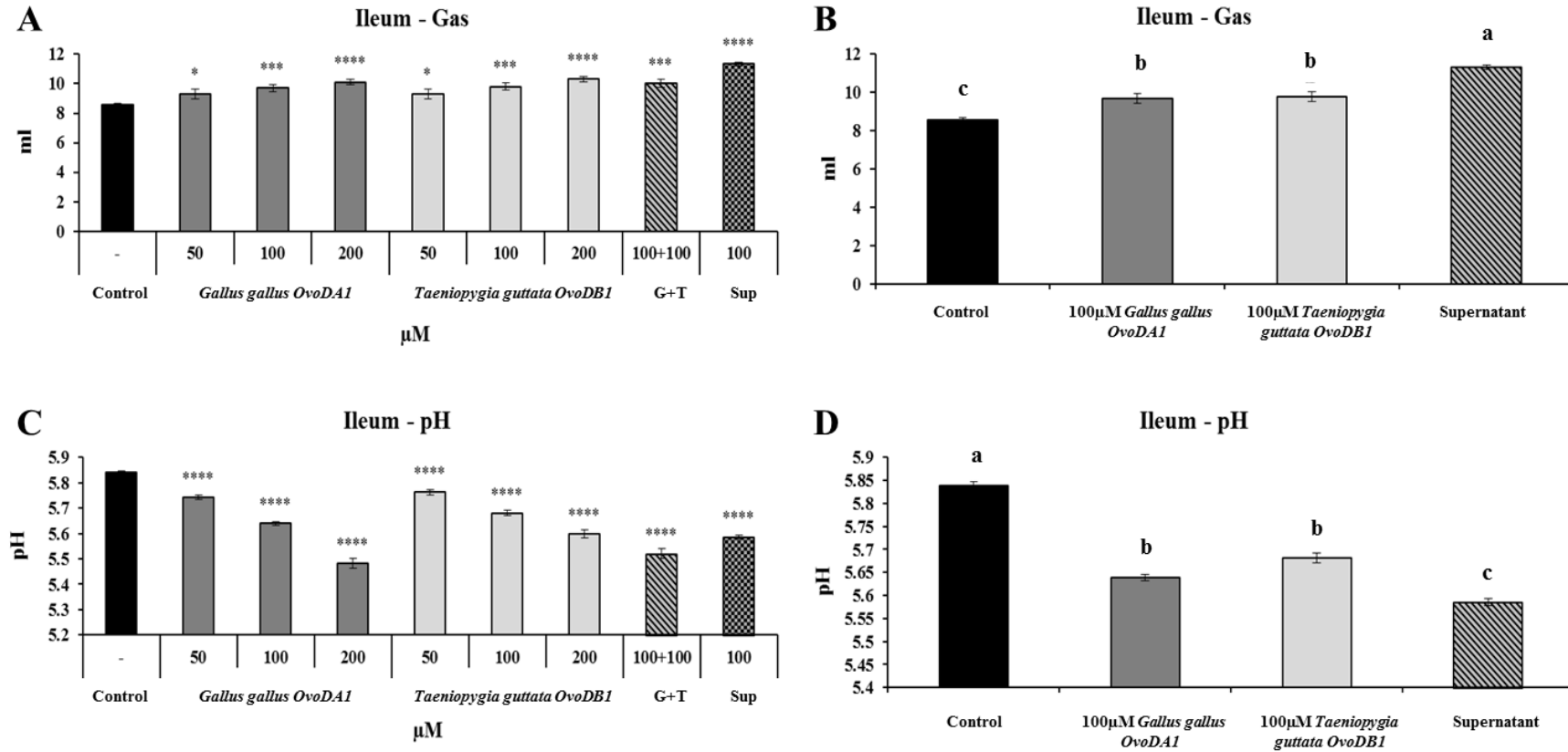


Figure 5.7: Effect of treatment on gas production and pH in the ileum digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on gas production (ANOVA, $P < 0.001$) (A, B) and pH (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D) are extracted from the data on the left and represent the data where 100μM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-c) where columns with different letters are significantly different to at least $P < 0.05$.

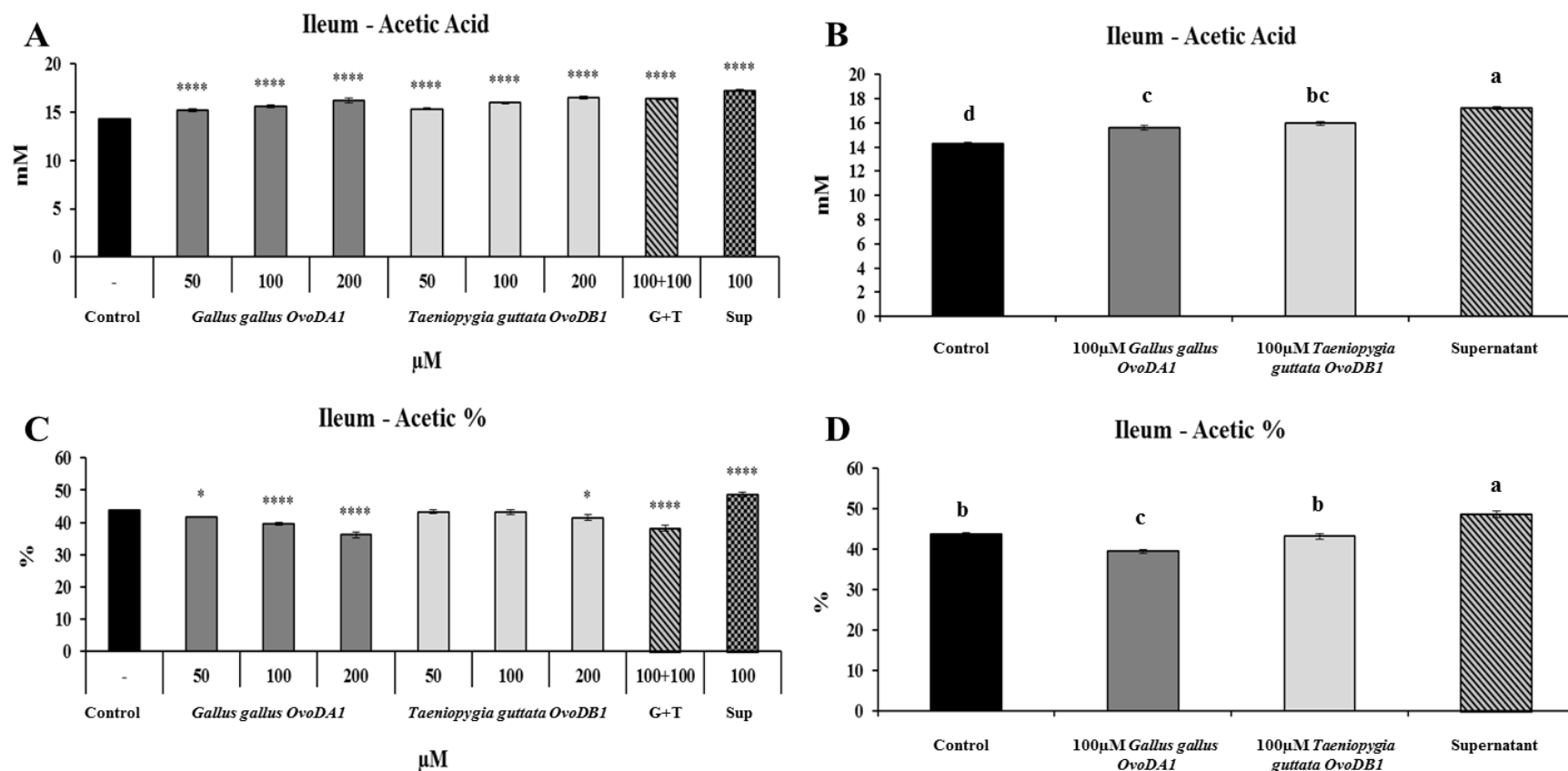


Figure 5.8: Effect of treatment on acetic acid in the ileum digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on acetic acid concentration (ANOVA, $P < 0.001$) (A, B) and percentage (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D) are extracted from the data on the left and represent the data where 100 μ M of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-c) where columns with different letters are significantly different to at least $P < 0.05$.

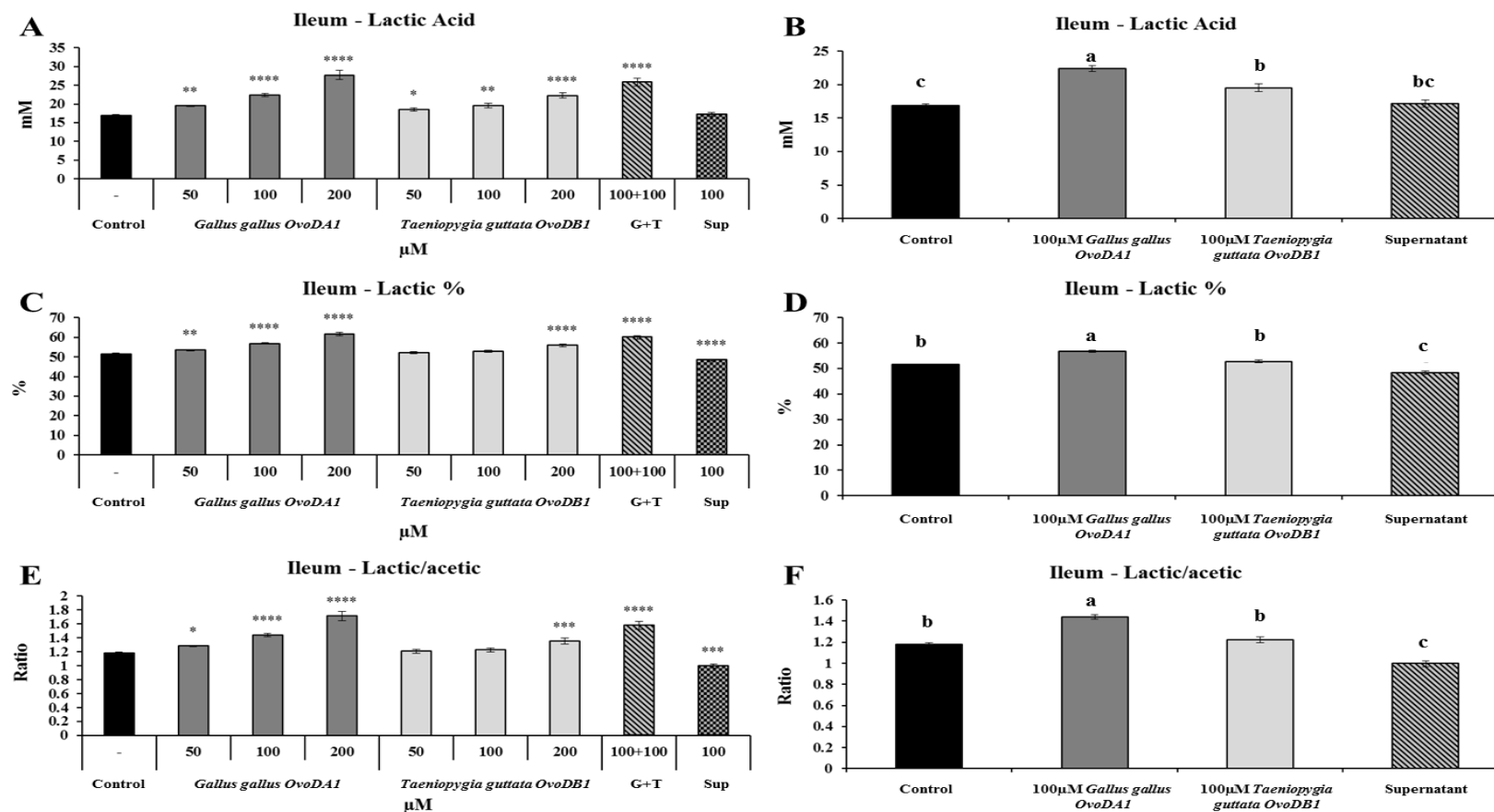


Figure 5.9: Effect of treatment on lactic acid in the ileum digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on lactic acid concentration (ANOVA, $P < 0.001$) (A, B) and percentage (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C, E) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D, F) are extracted from the data on the left and represent the data where 100μM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-c) where columns with different letters are significantly different to at least $P < 0.05$. The ratio of lactic and acetic acid (ANOVA, $P < 0.001$) illustrates the opposing changes in environment between peptide and supernatant treatment.

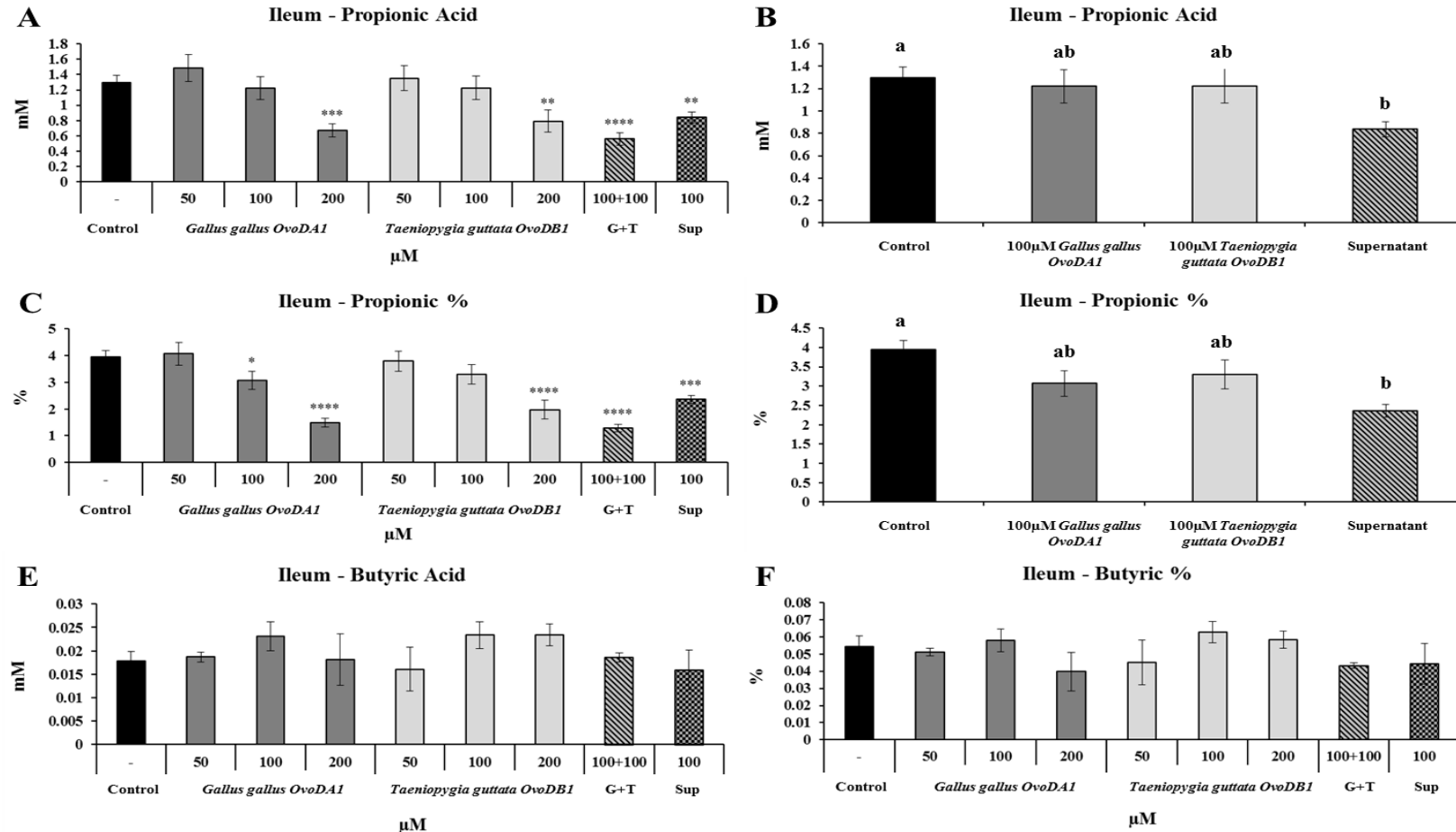


Figure 5.10: Effect of treatment on propionic acid and butyric acid in the ileum digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on propionic acid concentration (ANOVA, $P < 0.001$) (A, B) and percentage (ANOVA, $P < 0.001$) (C, D). No significant effect was observed for butyric acid concentration (ANOVA, $P = 0.564$) (E) or percentage (ANOVA, $P = 0.502$) (F). Graphs A, C, E and F show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs B and D are extracted from the data to their left and represent the data where 100µM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a,b) where columns with different letters are significantly different to at least $P < 0.05$.

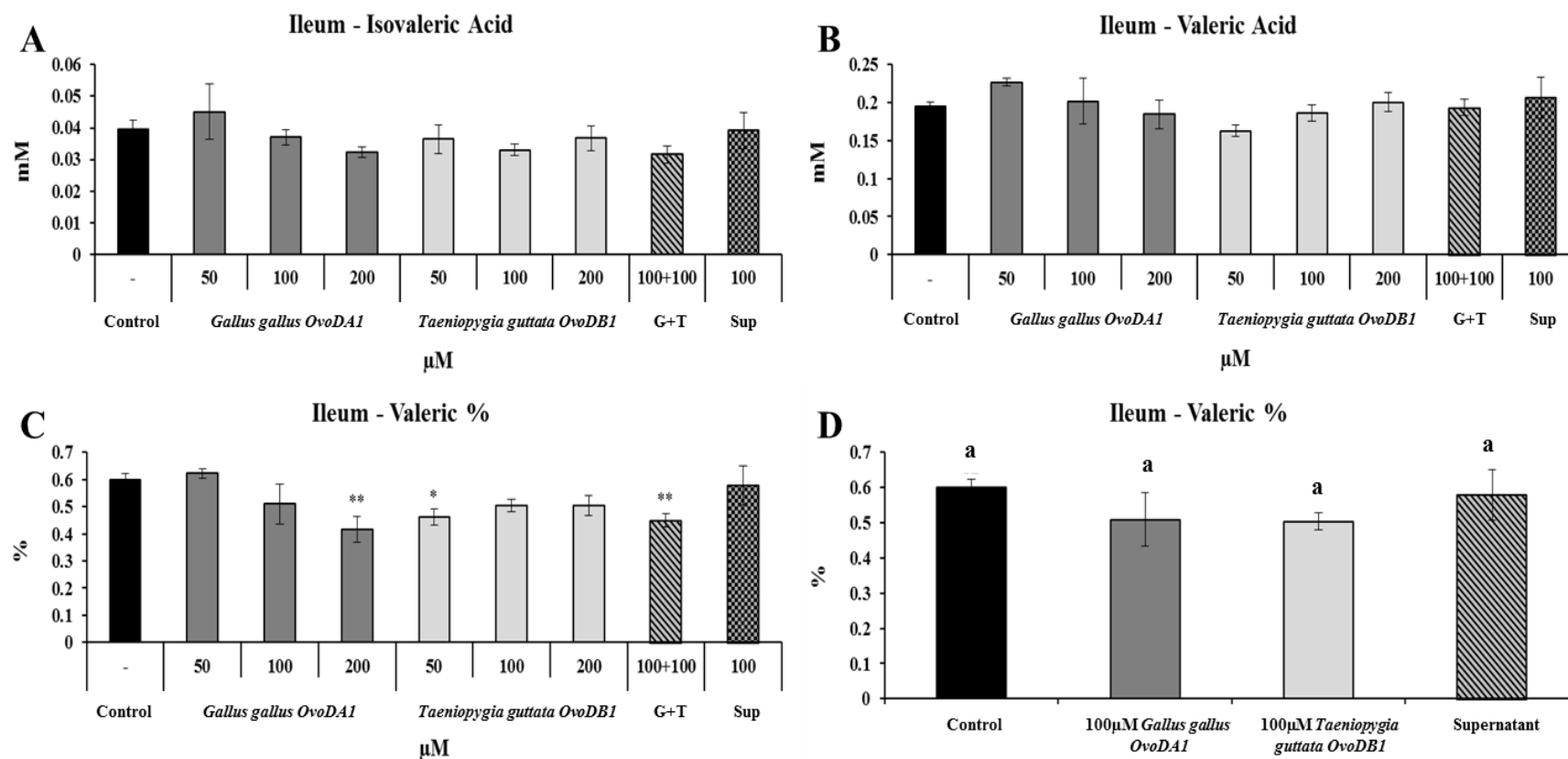


Figure 5.11: Effect of treatment on isovaleric and valeric acid in the ileum digesta (n=5, mean \pm sem). Analysis of variance showed no significant effect on isovaleric acid concentration (ANOVA, $P=0.479$) (A) or valeric acid concentration (ANOVA, $P=0.317$) (B). However the percentage of valeric acid in the ileum digesta was significantly altered (ANOVA, $P=0.007$) (C, D). Graphs on A-C show data for all treatments. The significance where indicated is in comparison with the control; $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****). Graph D is extracted from the data in C and represents the data where 100μM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a) where columns with different letters are significantly different to at least $P<0.05$.

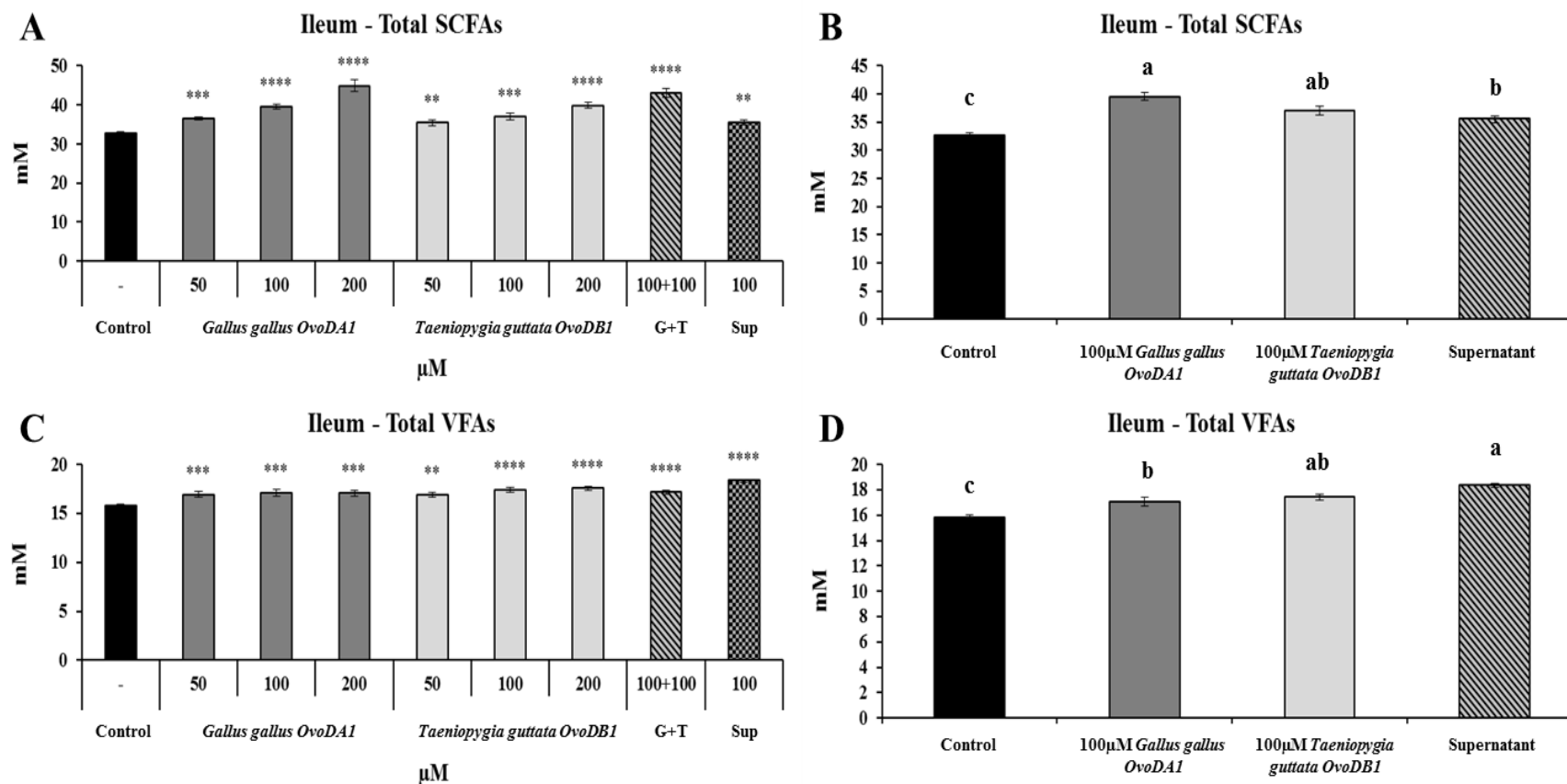


Figure 5.12: Effect of treatment on total short chain fatty acid (SCFAs) and volatile fatty acids (VFAs) in the ileum digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on total SCFA (ANOVA, $P < 0.001$) (A, B) and VFA concentration (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D) are extracted from the data on the left and represent the data where 100µM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-c) where columns with different letters are significantly different to at least $P < 0.05$.

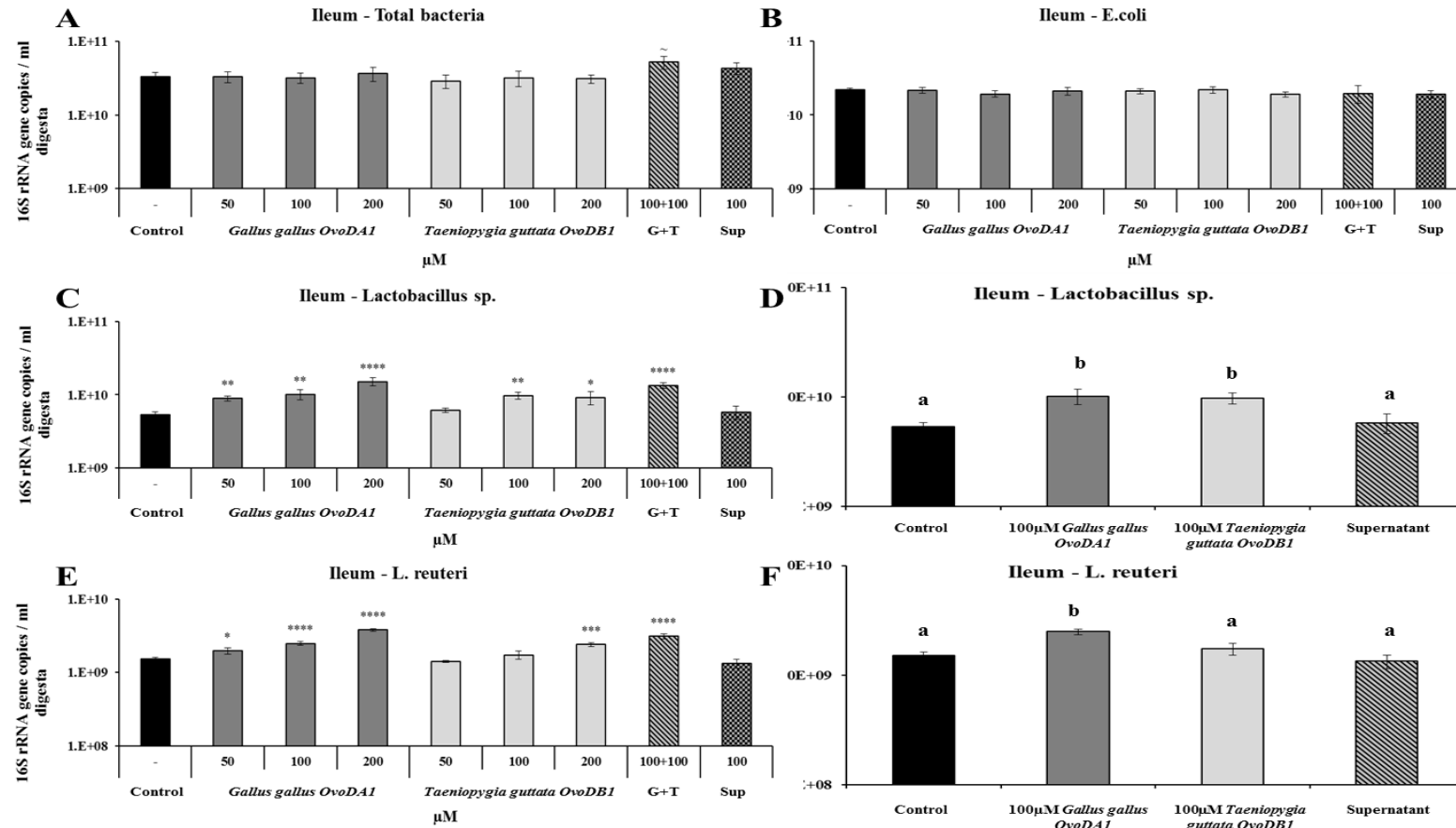


Figure 5.13: Effect of treatment on total bacterial numbers, *E. coli*, *Lactobacillus* species and *Lactobacillus reuteri* in the ileum digesta (n=5, mean \pm sem). Analysis of variance showed no significant effect on total bacteria (ANOVA, $P=0.413$) (A) or *E. coli* (ANOVA, $P=0.354$) (B). However the total number of *Lactobacillus* species in the ileum digesta was significantly altered (ANOVA, $P<0.001$) (C, D) with significant effects being observed in *L. reuteri* numbers in particular (ANOVA, $P<0.001$) (E, F). The significance where indicated is in comparison with the control; $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****). Graphs D and F are extracted from the data to their left and represent the data where 100µM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-b) where columns with different letters are significantly different to at least $P<0.05$.

Taeniopygia guttata OvoDB1 at 100 μ M was significant from the control however it was not significantly different to the supernatant control. Treatment also had a significant effect on the percentage of lactic acid in the *ex vivo* ileum (ANOVA, $P < 0.001$); lactic acid significantly increased when treated with 100 μ M *Gallus gallus* OvoDA1 however the supernatant control resulted in a significant reduction in % lactic acid (Figure 5.9C and 5.9D). These significant changes in acetic acid and lactic acid (ANOVA, $P < 0.001$) are shown as a ratio in Figures 5.9E and 5.9F for clarity. A significant effect was observed on propionic acid concentration (ANOVA, $P < 0.001$) (Figure 5.10A) and percentage (ANOVA, $P < 0.001$) (Figure 10C). However when individual doses were compared neither *Gallus gallus* OvoDA1 or *Taeniopygia guttata* OvoDB1 were significant from either the negative or supernatant controls when included in the feed at 100 μ M (Figure 5.10B and 5.10D). Butyric acid concentration and percentage also did not significantly differ due to treatment (ANOVA, $P = 0.564$ and $P = 0.502$ respectively) (Figure 5.10E and 5.10F). Isovaleric acid concentration did not significantly differ due to treatment (ANOVA, $P = 0.479$) (Figure 5.11A) nor did valeric acid (ANOVA, $P = 0.317$) (Figure 5.11B). When valeric acid was analysed as a percentage of the total SCFAs a significant difference was observed (ANOVA, $P = 0.007$). Further analysis revealed that this significance was only the highest (200 μ M) concentrations of *Gallus gallus* OvoDA1 ($P < 0.01$) and *Taeniopygia guttata* OvoDB1 ($P < 0.01$). Treatment had a significant effect on total short chain fatty acids (SCFA) (ANOVA, $P < 0.001$) (Figure 5.12A). Although both peptide treatment and supernatant resulted in an increase in SCFAs, treatment with 100 μ M *Gallus gallus* OvoDA1 resulted in significantly more SCFAs than the supernatant alone (Figure 5.12B). Total volatile fatty acids also significantly increased (ANOVA, $P < 0.001$) (Figure 5.12C) however both the peptide treatments and supernatant behaved similarly (Figure 5.12D). Isobutyric and 2-methyl-butyric acid were not measured in the ileum as they are not major components in this region.

The total number of bacteria in the ileum did not significantly differ for any of the treatments (ANOVA, $P = 0.413$) (Figure 5.13A) nor did total *Escherichia coli* numbers in any of the treatments (ANOVA, $P = 0.354$) (Figure 5.13B). Total

Lactobacillus species significantly increased (ANOVA, $P < 0.001$) (Figure 5.13C), and when 100 μM treatments were analysed separately to account for background fungal components they significantly increased compared to both the supernatant and the negative control (Figure 5.13D). The supernatant did not significantly differ from the negative control suggesting the increase in *Lactobacillus* was due to the peptide (Figure 5.13D). The level of significance observed in the ANOVA of this data is due to a highly significant increase in *Lactobacillus* species when 200 μM *Gallus gallus* OvoDA1 was used ($P < 0.0001$). In particular *Lactobacillus reuteri* showed a significant increase when recombinant peptide was administered (ANOVA, $P < 0.001$) (Figure 5.13C), this was significant when compared with the supernatant (Figure 5.13D).

5.4.4.2. Caeca

All treatments including the supernatant control resulted in an increase in the amount of caecal gas (ANOVA, $P < 0.001$) (Figure 5.14A and 5.14B) and overall there was a reduction in caecal pH (ANOVA, $P < 0.001$) (Figure 5.14C), however the greatest pH reduction was observed with the control supernatant (Figure 5.14D). There was an overall increase in acetic acid concentration (ANOVA, $P < 0.001$) (Figure 5.15A) and percentage (ANOVA, $P < 0.001$) (Figure 5.15C) in the caeca. However this observation was observed for both the peptide and supernatant treatments (Figure 5.15B and 5.15D). Both propionic acid (ANOVA, $P < 0.001$) (Figures 5.16A-D) and butyric acid (ANOVA, $P < 0.001$) (Figures 5.17A-D) concentration in the caeca increased under all test conditions; however this was not peptide specific. Isobutyric acid (Figures 5.18A) and 2-methyl-butyric acid (Figures 5.18C) showed a significant decrease in the caecal model (ANOVA, $P < 0.001$; $P = < 0.001$), independent of whether peptide or supernatant was added (Figure 5.18B and 5.18D). There was a significant effect on valeric acid concentration (ANOVA, $P < 0.001$) and percentage (ANOVA, $P = 0.002$) (Figures 5.19A and 5.19C) however this did not differ upon dosing with peptide or supernatant control (Figures 5.19B and 5.19D). Isovaleric acid decreased overall (ANOVA, $P < 0.001$) but this was not peptide specific (Figure 5.19E and 5.19F). Total short chain fatty acids increased overall (ANOVA, $P < 0.01$) (Figures

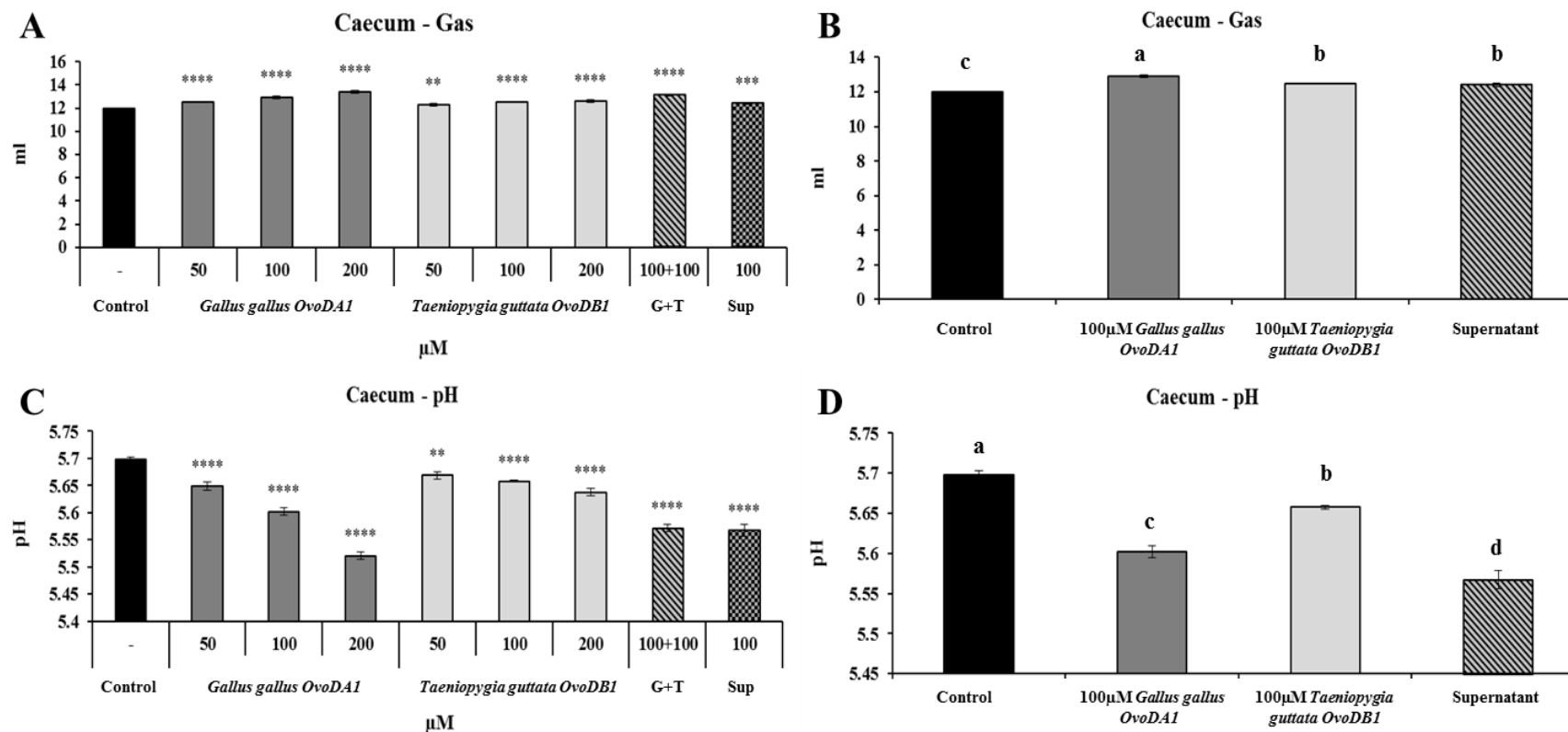


Figure 5.14: Effect of treatment on gas production and pH in the caecal digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on gas production (ANOVA, $P < 0.001$) (A, B) and pH (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D) are extracted from the data on the left and represent the data where 100μM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-d) where columns with different letters are significantly different to at least $P < 0.05$.

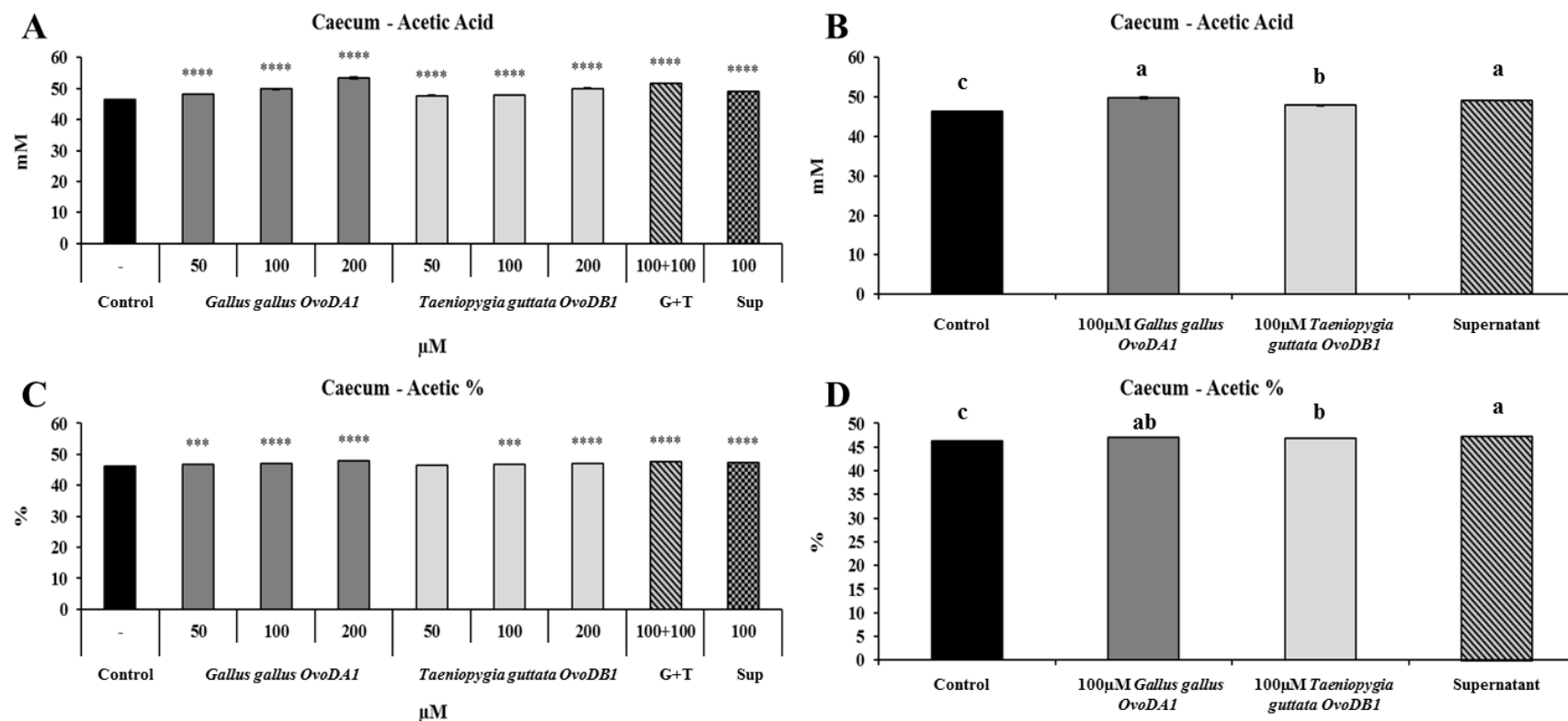


Figure 5.15: Effect of treatment on acetic acid in the caecal digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on acetic acid concentration (ANOVA, $P < 0.001$) (A, B) and percentage (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D) are extracted from the data on the left and represent the data where 100 μ M of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-c) where columns with different letters are significantly different to at least $P < 0.05$.

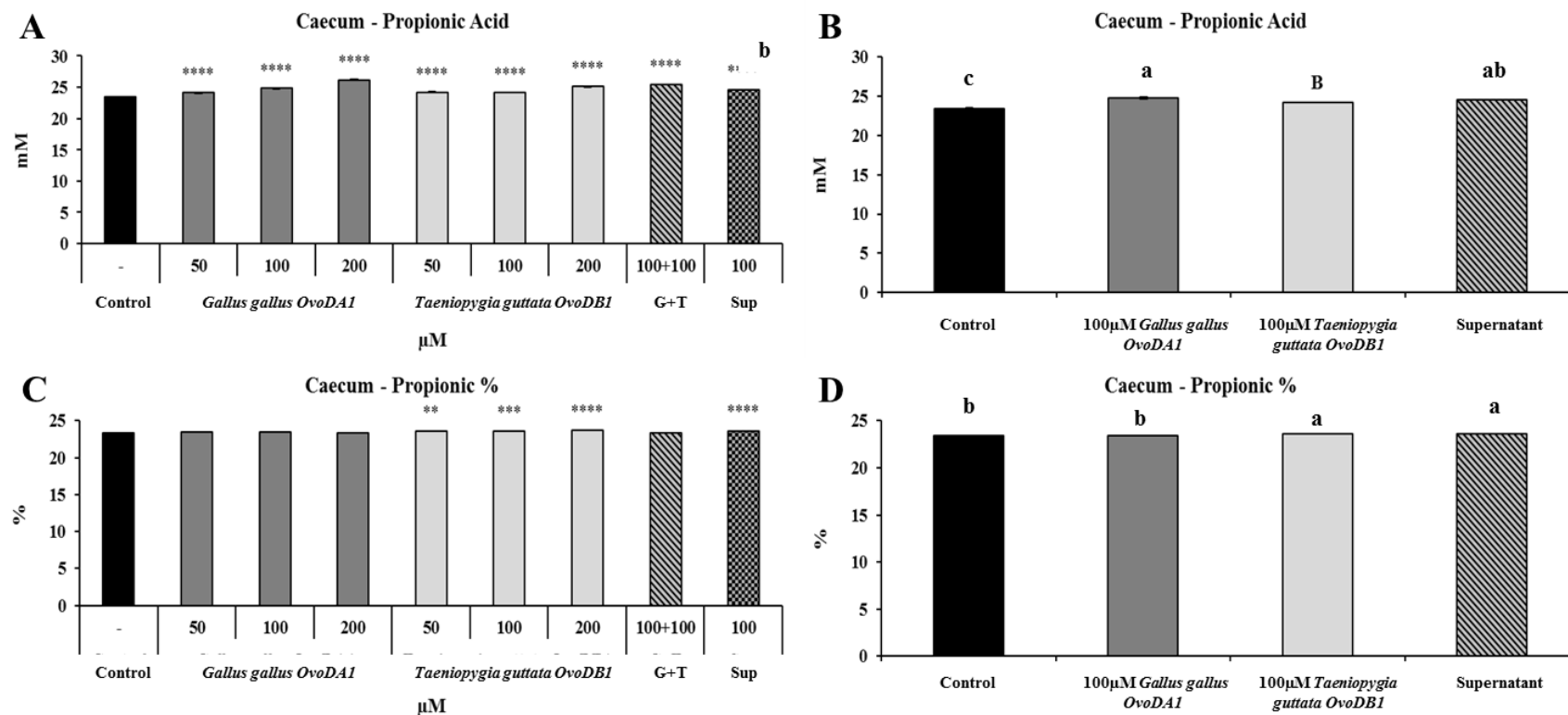


Figure 5.16: Effect of treatment on propionic acid in the caecal digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on propionic acid concentration (ANOVA, $P < 0.001$) (A, B) and percentage (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D) are extracted from the data on the left and represent the data where 100µM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-c) where columns with different letters are significantly different to at least $P < 0.05$.

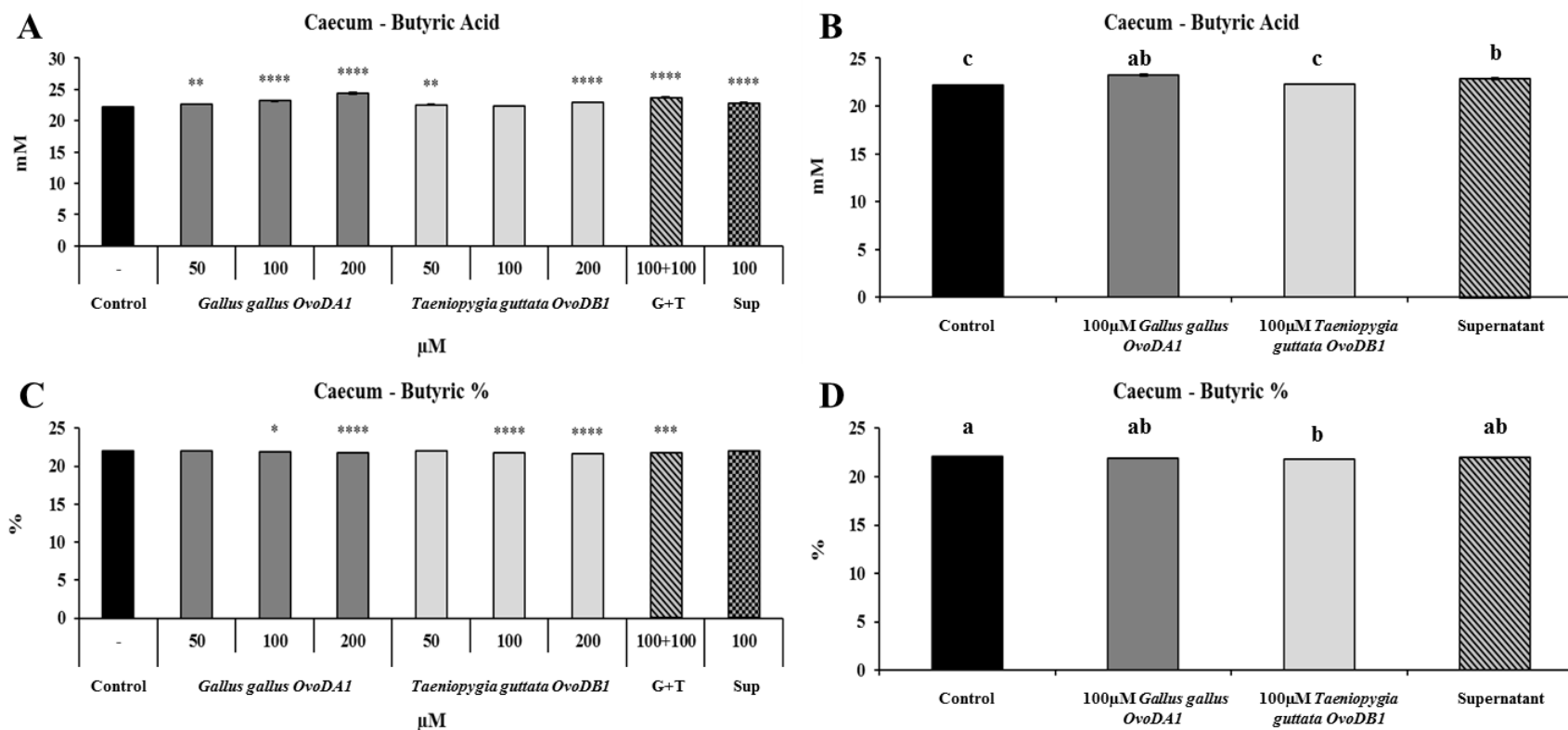


Figure 5.17: Effect of treatment on butyric acid in the caecal digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on butyric acid concentration (ANOVA, $P < 0.001$) (A, B) and percentage (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D) are extracted from the data on the left and represent the data where 100 μ M of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-c) where columns with different letters are significantly different to at least $P < 0.05$.

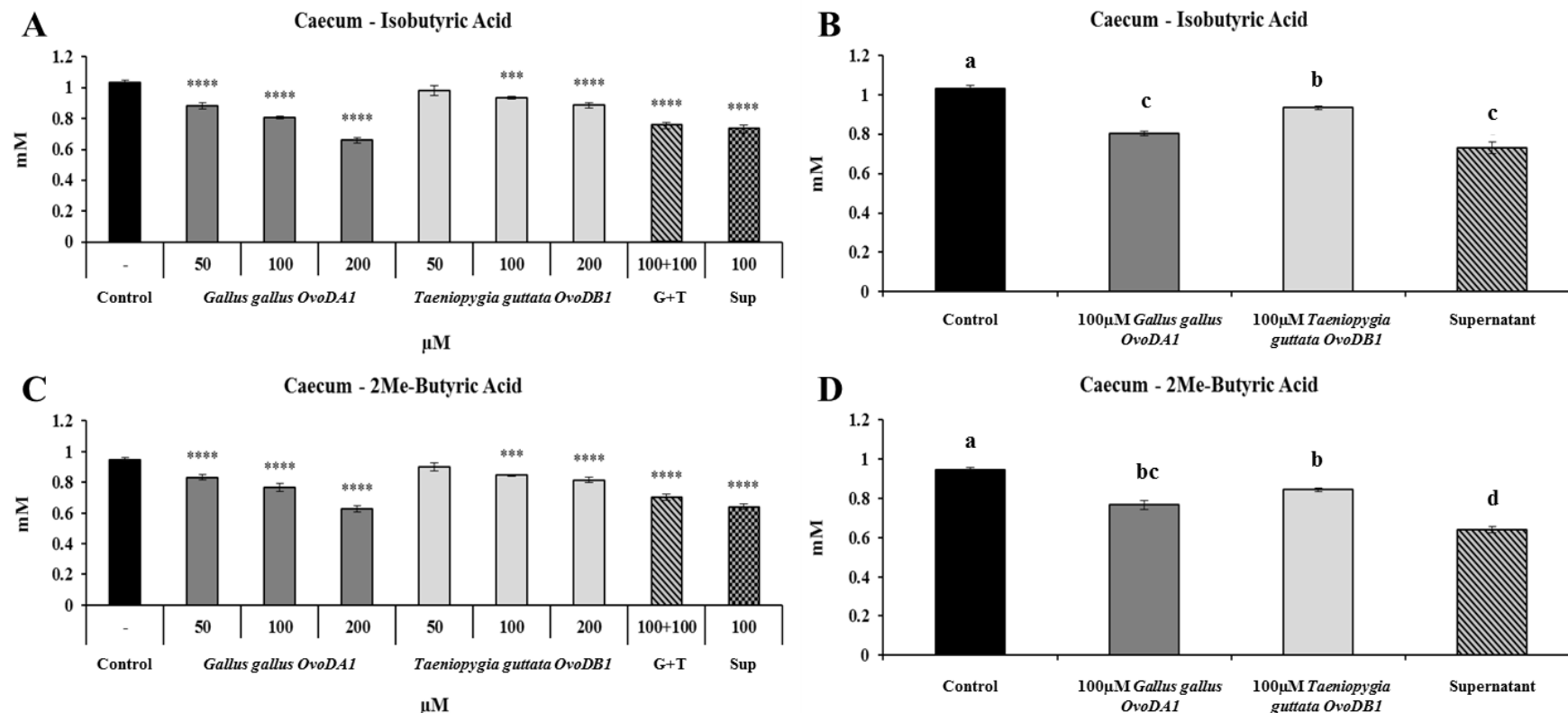


Figure 5.18: Effect of treatment on isobutyric and 2Me-butyric acid in the caecal digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on isobutyric acid (ANOVA, $P < 0.001$) (A, B) and 2Me-butyric acid concentration (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D) are extracted from the data on the left and represent the data where 100 μ M of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-d) where columns with different letters are significantly different to at least $P < 0.05$.

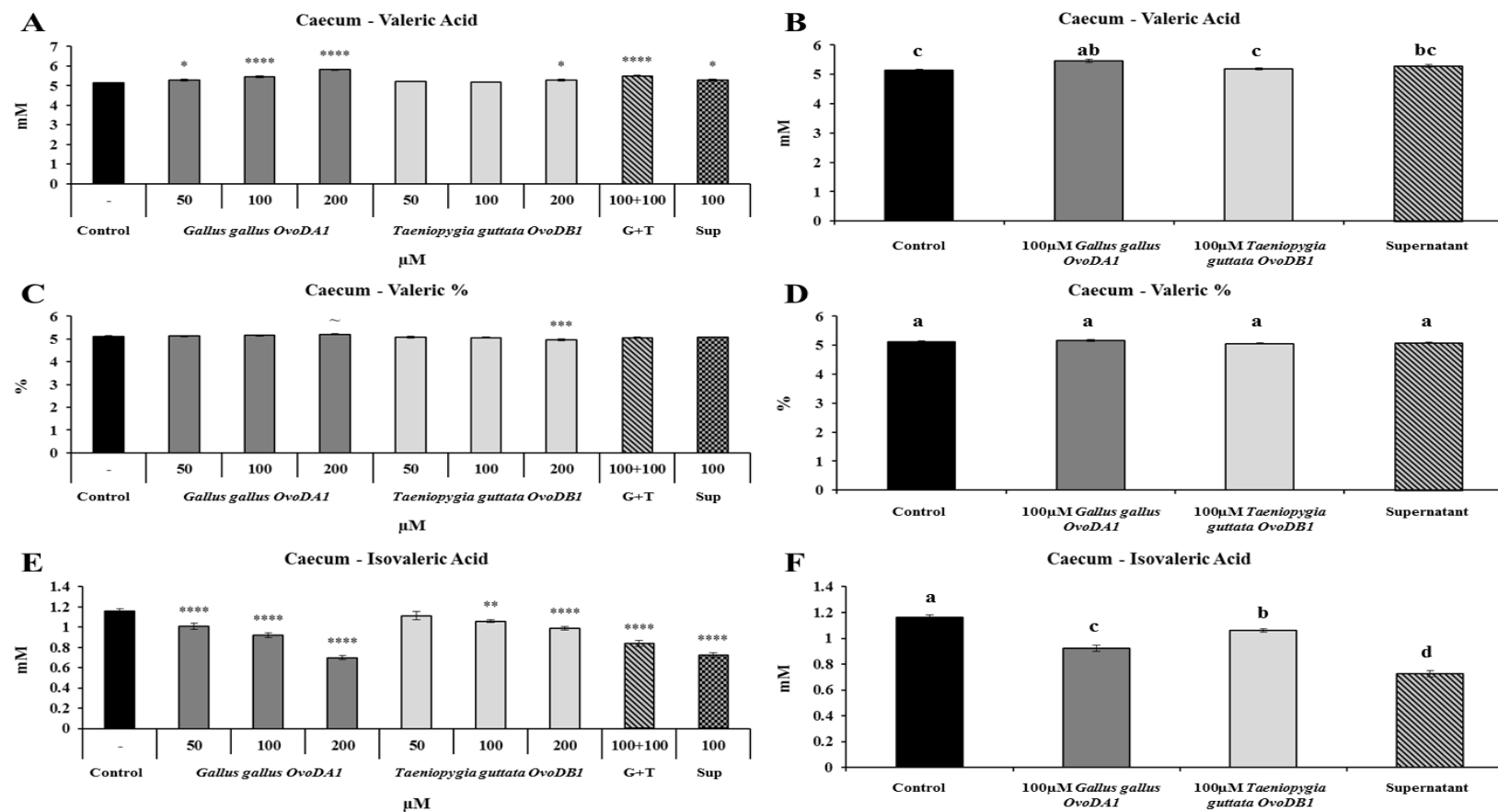


Figure 5.19: Effect of treatment on valeric and isovaleric acid in the caecal digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on both valeric acid concentration (ANOVA, $P < 0.001$) (A, B) and percentage ($P = 0.002$) and isovaleric acid concentration (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C, E) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D, F) are extracted from the data on the left and represent the data where 100μM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-d) where columns with different letters are significantly different to at least $P < 0.05$.

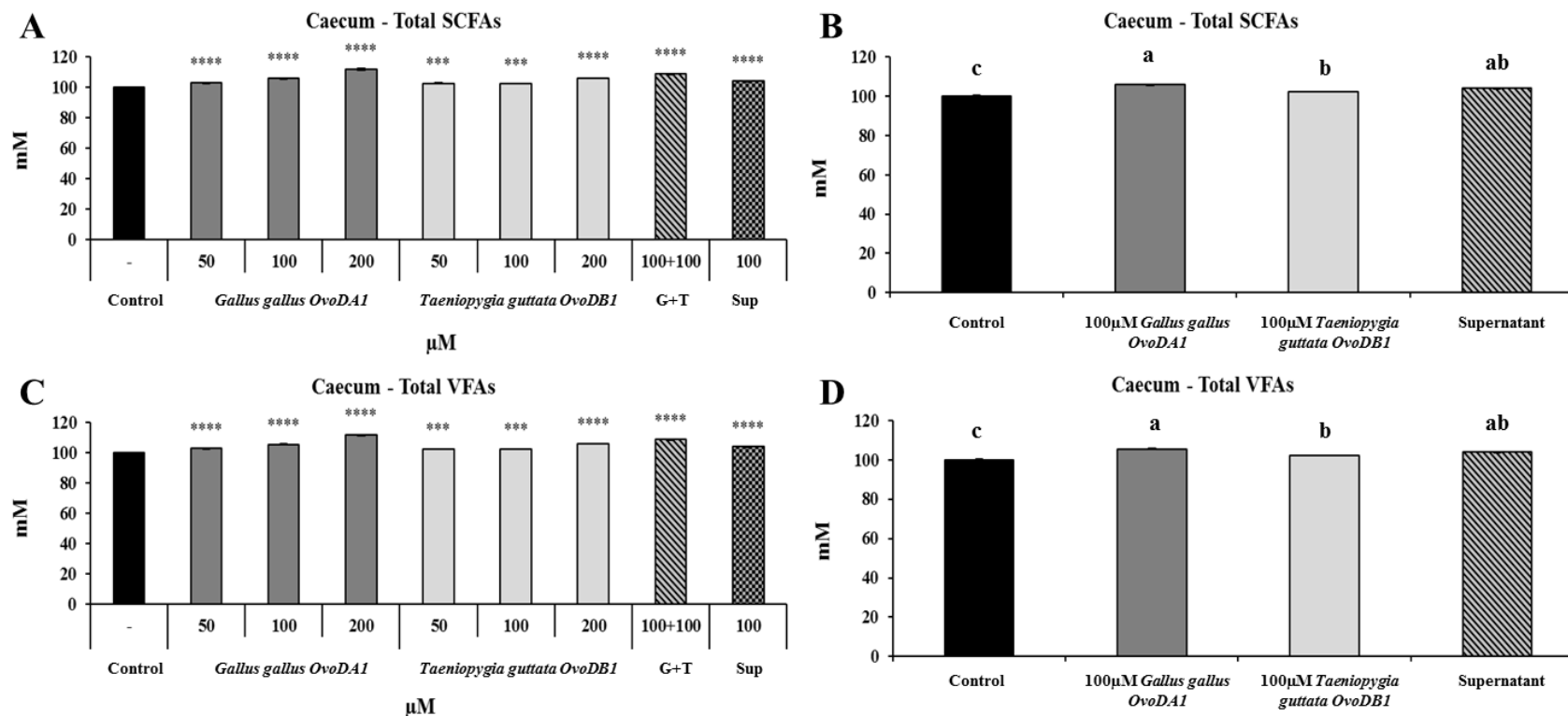


Figure 5.20: Effect of treatment on total short chain fatty acid (SCFAs) and volatile fatty acids (VFAs) in the caecal digesta (n=5, mean \pm sem). Analysis of variance showed a significant effect on total SCFA (ANOVA, $P < 0.001$) (A, B) and VFA concentration (ANOVA, $P < 0.001$) (C, D). Graphs on the left hand side (A, C) show data for all treatments. The significance where indicated is in comparison with the control; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Graphs on the right (B, D) are extracted from the data on the left and represent the data where 100µM of each peptide was used and where the supernatant concentration was equal. This therefore represents a more appropriate comparison than comparing all data as at this concentration the supernatant provides a weight/weight control for background fungal components. In this panel comparisons are represented by letters (a-c) where columns with different letters are significantly different to at least $P < 0.05$.

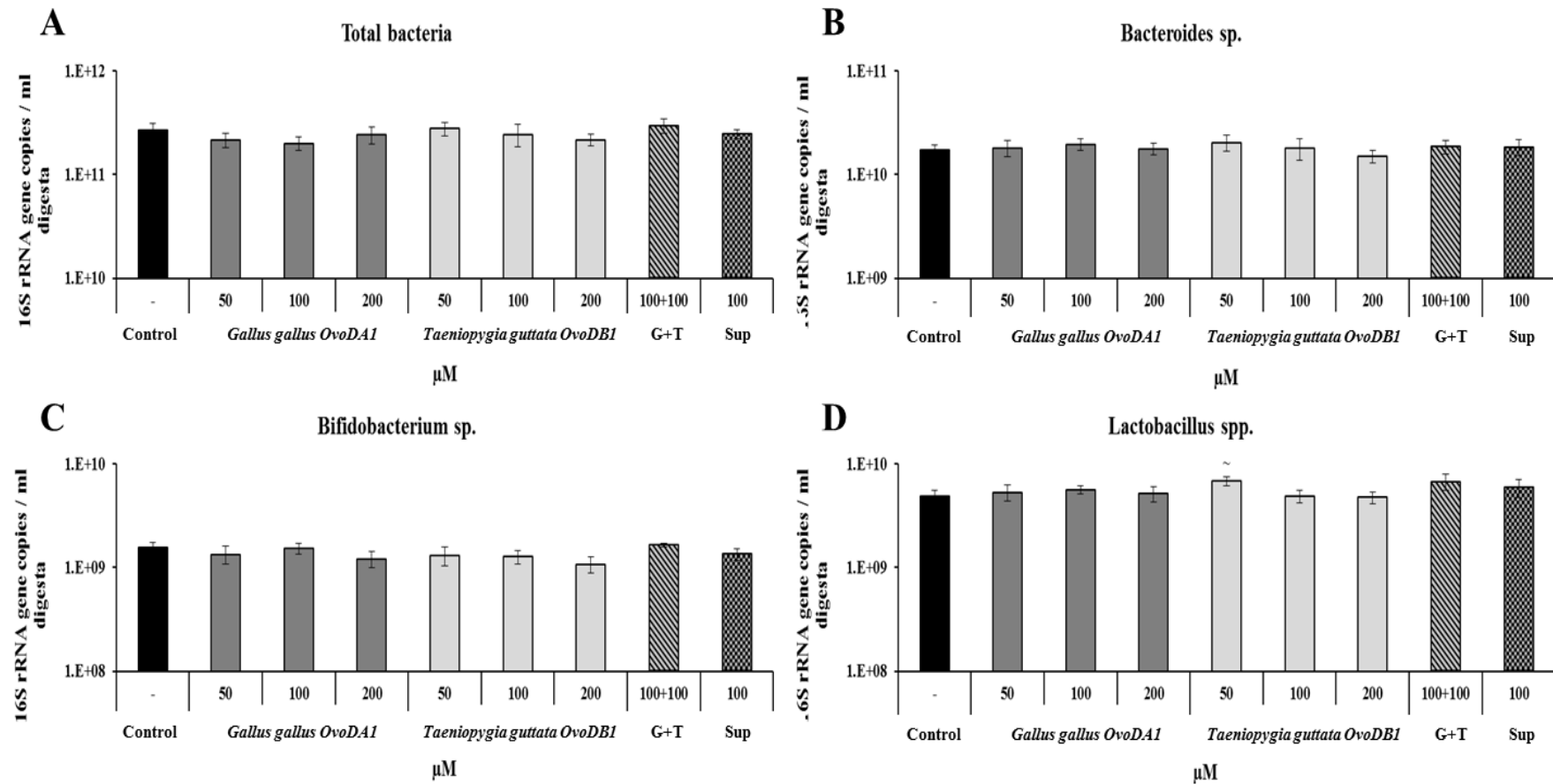


Figure 5.21: Effect of treatment on the total number of bacteria, total Bacteroides, Bifidobacterium and Lactobacillus species in the caecum digesta (n=5, mean ± sem). No significant effect was observed on total number of bacteria (ANOVA, $P=0.801$) (A), Bacteroides (ANOVA, $P=0.979$) (B), Bifidobacterium (ANOVA, $P=0.239$) or Lactobacillus species (ANOVA, $P=0.609$).

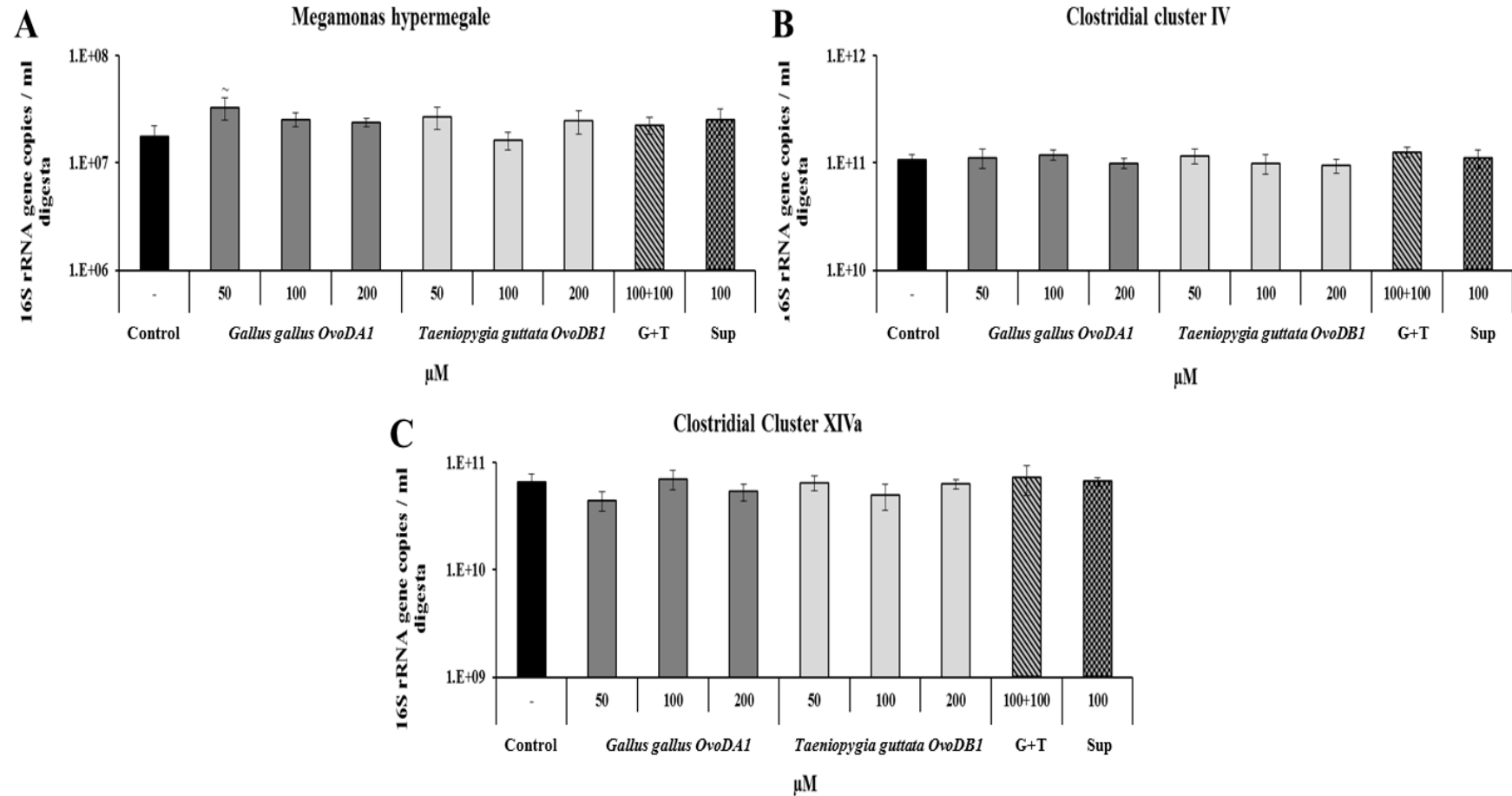


Figure 5.22: Effect of treatment on *Megamonas hypermegale*, Clostridial cluster IV and Clostridial cluster XIVa in the caecal digesta (n=5, mean \pm sem). No significant changes were observed in *Megamonas hypermegale* (ANOVA, $P=0.598$) (A), Clostridial cluster IV (ANOVA, $P=0.570$) (B) or Clostridial cluster XIVa (ANOVA, $P=0.370$) (C) in the caecal digesta.

5.20A) but peptide treatment did not differ from the supernatant control (Figure 5.20B). Volatile fatty acids were also significantly increased (ANOVA, $P < 0.001$) (Figure 5.20C), however again this was not peptide specific when compared with the supernatant control (Figure 5.20D). Lactic acid concentrations were measured but not shown as levels were extremely low as expected with caecal contents (Rinttila and Apajalahti, 2013).

No significant changes in total bacteria numbers (ANOVA, $P = 0.801$) (Figure 5.21A) or any specific species was measured in the caecal contents. Total *Bacteroides* (ANOVA, $P = 0.979$) (Figure 5.21B), *Bifidobacterium* (ANOVA, $P = 0.239$) (Figure 5.21C) and *Lactobacillus* (ANOVA, $P = 0.609$) (Figure 5.21D) species, *Megamonas hypermegale* (ANOVA, $P = 0.598$) (Figure 5.22A) and Clostridial clusters IV and XIVa (ANOVA, $P = 0.570$ and $P = 0.370$ respectively) (Figures 5.22B and 5.22C) measured using RT-qPCR were not significant.

5.5. Discussion

In support of an egg defence role, *Gallus gallus* OvoDA1 was previously shown to have antimicrobial activity against *E. coli* (Gong et al., 2010), an observation that was confirmed by Hervé et al (2014). The data outlined in this study also demonstrated antimicrobial activity of *Gallus gallus* OvoDA1 against laboratory-adapted and pathogenic *E. coli* (Figure 5.1A), and in agreement with the former publication (Herve et al., 2014), no activity was found against *Salmonella* serovars Enteritidis and Typhimurium (Table 5.1). However this study did demonstrate antimicrobial activity of *Gallus gallus* OvoDA1 against *S. aureus* (Figure 5.1B). Although it had been previously documented that *Gallus gallus* OvoDA1 did not possess antimicrobial activity against *S. aureus* (Herve et al., 2014) both the method used to measure antimicrobial activity and the strain differ. This is the first time activity has been recorded for a Gram-positive organism with an ovodefensin, and indeed for an organism other than *E. coli* demonstrating the need for more in depth analysis of the spectrum of activity of the ovodefensins.

For the first time this study examined the antimicrobial activity of two other avian members of the ovodefensin family, *Gallus gallus* OvoDB1 and *Anas platyrhynchos* OvoDA1. *Gallus gallus* OvoDB1 represents a member of the sub-family B cysteine motif (C-X3-C-X3-C-X11-C-X4-CC) whereas *Anas platyrhynchos* OvoDA1 contains the same sub-family A cysteine motif as *Gallus gallus* OvoDA1 (C-X5-C-X3-C-X11-C-X3-CC). Although *Gallus gallus* OvoDB1 possessed antimicrobial activity against *E.coli* DH5 α (35% reduction in CFU/ml) (Figure 5.2) it was not as potent as OvoDA1 from the same species at the same concentration (100 μ M) and no activity was recorded against APEC, *S. aureus* or either of the *Salmonella* strains tested in this study (Table 5.1). *Anas platyrhynchos* OvoDA1 demonstrated good activity against *E.coli* DH5 α (>80% reduction) (Figure 5.3) yet no activity was recorded against any of the other strains used in this study (Table 5.1). The antimicrobial results from this study suggest that the family is as diverse in its activity as it is in sequence which questions whether the sequence or perhaps spacing within the cysteine motifs of these molecules affects their ability to kill microorganisms, perhaps evolving to counter the specific challenges each organism faces. However both *Gallus gallus* and *Anas platyrhynchos* OvoDA1 are from the same sub-family suggesting that spacing alone does not define direct antimicrobial activity. Another possibility for differing levels of activity is charge which varies from +4 to +10 within the currently identified ovodefensin members. However when considering that *Gallus gallus* OvoDA1 has a charge of +7, *Gallus gallus* OvoDB1 a charge of +10 and *Anas platyrhynchos* OvoDA1 +6 charge alone does not seem to explain the differences in activity noted.

For use as a feed additive to be plausible the peptides need to be produced in large quantities economically. The company involved in this project (AB Vista) (<https://www.abvista.com/>) have a large amount of expertise in the recombinant feed additive field and their technology allowed both of these issues to be addressed through a fungal fermentation system. However the recombinant product produced needs to be active. *Gallus gallus* OvoDA1 was the most active in the *in vitro* tests conducted and was therefore used for production in this system. *Taeniopygia guttata*

OvoDB1 was identified as the avian ovodefensin differing most from *Gallus gallus* OvoDA1 in terms of sequence, peptide length and spacing in Chapter 3 and it was therefore decided that it would also be produced in the fungal system. Both neat recombinant *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1 showed potent activity against *E.coli* DH5 α in the antibacterial assay. *Gallus gallus* OvoDA1 achieved an 83% reduction in CFU/ml and *Taeniopygia guttata* OvoDB1 reduced *E.coli* DH5 α by 98%. These results show that recombinant ovodefensin peptides have antibacterial activity and could potentially be used as a feed additive.

As *Gallus gallus* OvoDA1 was the most potent ovodefensin in these experiments it was tested against a pathogen of significant interest to the poultry industry, *Campylobacter jejuni*. *Campylobacter* is the leading cause of food poisoning in the UK with *Campylobacter* poisoning results in approximately 100 deaths each year and costs the UK economy about £900 million (WHO, 2011). *Gallus gallus* OvoDA1 only resulted in a 10% reduction in the wild type strain of this organism. However this particular strain is known to possess genes which are thought to confer resistance to positively charged antimicrobial peptides (unpublished) and when *Gallus gallus* OvoDA1 was tested against a mutant strain with these genes removed an impressive 41% reduction was observed. This is an important observation as only approximately 50% of *Campylobacter* strains possess these particular genes (Mark Stevens, March 2014, personal communication) and therefore this ovodefensin peptide may be effective against other *Campylobacter* strains too. However further testing against a larger panel of *Campylobacter* strains would need to be undertaken to determine the true effectiveness of this peptide as it is possible that other genes in different strains may also convey resistance to direct antibacterial activity of charged peptides.

If administered as a feed additive then the recombinant peptide would need to work under a variety of environmental conditions. *Gallus gallus* OvoDA1 was therefore assessed in further detail. The pH of the target region of the intestine, the small intestine is around pH 6.4 (Mabelebele et al., 2014) so pH was chosen as a factor for study. Altering pH did not significantly affect the ED50 of *Gallus gallus* OvoDA1

(Figure 5.5A) however it is clear from the graph that overall potency of the peptide did diminish at pH 6.4. Although there was a small reduction in potency it is promising that the activity of the peptide was not drastically reduced suggesting that the peptide is not inactivated at lower pH.

Salt sensitivity of classical mammalian defensins such as mouse β -defensin 1 (Bals et al., 1998) has been well documented and it is suggested that this is a feature common to all defensin molecules (Ganz, 2003), it was therefore important to test the effect of salt on ovodefensin activity. Again *Gallus gallus* OvoDA1 was chosen for this work. Salt concentrations were chosen to approximately reflect the natural environment of the peptide, the egg (50 mM) (Bell and Freeman, 1984), the standard assay conditions (100 mM) and physiological salt concentrations of the chicken milieu (150 mM) (Bell and Freeman, 1984). Salt concentration did not significantly affect the ED50 value however it is clear that salt concentration did significantly affect the maximal potency of the peptide (Figure 5.5B) resulting in reduction in overall kill of 32% at the highest concentration tested and a maximum reduction in killing of 58% at 16.7 μ M.

The metabolic state of APEC did not affect antibacterial activity of *Gallus gallus* OvoDA1 suggesting that the mode of action of this peptide does not require the cell to be replicating. This is consistent with the proposed model of defensin activity which involves electrostatic interaction with the cell membrane. The diminished activity of ampicillin in this assay demonstrates the fact that the bacterial cells were in metabolically static state and unable to replicate as this is a vital process for ampicillin activity because it interferes with cell wall synthesis (Kong et al., 2010). The effect of metabolic state would likely have been clearer had *E.coli* DH5 α been used as the peptide is more potent against this strain. However the results suggest that metabolic state is not important for activity which widens the potential use of this peptide if it was to be considered for purposes other than a feed additive.

Some defensins show antiviral activity and it was therefore of interest to see if the ovodefensins possessed this property. A/WSN/33 is a human H1N1 strain isolated

post mortem from a casualty of the 1918 Spanish influenza pandemic (Taubenberger, 2006). This particular strain does not have the ability to infect birds however it can be used as a model organism as some H1N1 do possess this ability and it was necessary to choose a strain that was able to infect the MDCK cells commonly used in this assay. None of the peptides tested were able to reduce the number of viral plaques formed in this assay. This does not rule out the ability of ovodefensins to kill viruses as antiviral peptides have differing spectrums of activity and it may be that ovodefensins are effective against other viruses. Importantly it was also noted that, at least under visual inspection, no toxicity was observed to the eukaryotic MDCK cells used in the assay with cell monolayers remaining intact in the presence of peptide when no virus was added. As viruses are not the primary target for a feed additive it was decided that antiviral work would be taken no further.

As some defensins are documented to have immunomodulatory activities (see Chapter 1, Figure 1.4) a small macrophage infection assay was carried out to examine this function. *Gallus gallus* OvoDA1 did not improve the ability of HD11 macrophage-like cells to phagocytise *Salmonella*. However this was a pilot assay and the mechanisms of immunomodulatory activity are complex and likely to require other effector molecules which will be discussed in more detail in Chapter 7. This assay can therefore only tell us that the peptide is unable to directly activate HD11 cells under these conditions. It cannot rule out immunomodulatory activities; however a vast amount of work would be required to evaluate these modes of action in more detail that could not be achieved in the timescale of this thesis.

The *ex vivo* gut model produced some very promising results, particularly in the ileum, and it would appear that ovodefensins, in particular *Gallus gallus* OvoDA1 are able to modulate the gut microbiome and environment in a dose dependent manner. Although no major significant changes when compared to the supernatant were observed in the caeca the changes in the ileum were indicative of a positive shift in the microbiome and correlate well with Alimetrix's in-house performance indexes. In particular the increase in *Lactobacillus* species (Figure 5.13D) is promising. It is generally accepted that this genus is associated with good gut health,

and *L. reuteri* (Figure 5.13F) is currently used as a probiotic to this end (Spinler et al., 2008). It was particularly promising that this effect was specific to the treatments containing peptide and is therefore unlikely to be due to any residual background fungal components. The increase in lactic acid in this region (Figure 5.9A) also supports this change in the microbial community. Other indicators of good gut health such as a reduction in pH and an increase in butyric acid (Rinttila and Apajalahti, 2013) were also recorded in the study; however, as the control supernatant had the same effect we are unable to attribute this change to the peptide. This matters if trying to determine the exact effect of the ovodefensin peptide in gut modulation however the aim of this PhD thesis was to identify a product which could be used as a feed additive to modulate gut health. To this end the 'ovodefensin product' showed a number of promising changes to the parameters measured and the lack of obvious toxicity observed in the viral and macrophage infection assay suggest that the product would be safe. Therefore *Gallus gallus* OvoDA1 and *Taeniopygia guttata* OvoDB1 were used for large scale production and *in vivo* analysis; the results of this trial are documented in Chapter 6.

Chapter 6

Ovodefensin feed additives

6.1. Introduction

The efficacy and cost effectiveness of traditional antibiotics made them a popular choice for controlling enteric disease and promoting growth in livestock (Bedford, 2000). As discussed in Chapter 1 many concepts, including the use of antimicrobial peptides (AMPs), have been suggested as potential substitutes for the recently outlawed antibiotic compounds. However, discovery of novel candidates and cost of production has proved problematic (Hancock and Sahl, 2006). The identification of a large number of AMPs and the use of the recombinant production system outlined in this thesis has begun to address these issues and the *ex vivo* gut model outlined in chapter 5 revealed the potential for the use of AMPs as intestinal modulators. It is also clearly desirable that new strategies for disease control in animals have no ties to human treatment so that if resistance did develop, although unlikely, there would not be an effect on human medicine. Despite promising results *in vitro* there has been few trials exploring the use of AMPs *in vivo* (Kogut et al., 2010, Kogut et al., 2013) and as such there is clearly a requirement to test their efficacy in poultry.

In order to assess if the inclusion of AMPs in feed are having a beneficial effect on the growth and health of poultry several parameters were assessed. The major indicator of a successful trial is the increase in growth performance as this was a key outcome of the previously used antibiotic growth promoters and directly affects the profitability of the industry (Bedford, 2000, Miles et al., 2006). To assess this body weight was recorded, and as birds were fed *ab libitum* feed intake was also recorded to calculate the food conversion ratio (FCR) and prevent false positives if there was an increase in feed intake.

If an effect was observed, understanding the mechanisms behind it would be extremely complex as many factors influence growth including gut integrity, intestinal microbiota and the health/immune status of the bird (Whittow, 1999). Examining all these factors in detail was not possible due to constraints on time and resources however it was hypothesised that the microbiota of the bird may be affected both directly and indirectly by ovodefensins due to their known antibacterial

activity and proposed immunomodulatory capabilities. If this was the case then examining the microbial composition of the gastrointestinal tract (GIT) and environmental factors including pH, gas production and bacterial metabolites are a good indicator of such effects (Apajalahti, 2005). Additionally there is evidence to suggest that the bacterial composition of the GIT and in particular the resulting fermentation products directly affect the performance of the bird either by affecting the immune response e.g. increasing endogenous defensin expression (Sunkara et al., 2011) or by altering the efficiency of nutrient absorption (Rinttila and Apajalahti, 2013).

It is important to note, however, that the approach taken aimed first and foremost to determine if the in-feed inclusion of ovodefensins improve bird growth as determined by body weight. The experiment also aimed elucidate some of the mechanisms behind a change in growth performance; however the trial would still be deemed successful if an increase in body weight was observed in the absence of a change in the GIT microbiota or environment.

6.2. Objectives

To test the hypothesis that the inclusion of ovodefensin peptide in poultry feed can improve growth performance of broilers through the modulation of the gut microbiome and environmental profile e.g short chain fatty acid profile.

6.3. Methods

Gallus gallus OvoDA1 and *Taeniopygia guttata* OvoDB1 were included in the feed at 0.0047, 0.047 and 0.47 g/kg and 0.0045, 0.045 and 0.45 g/kg respectively. Peptide was produced using a recombinant fungal system as outline in 2.14. The negative control for this experiment was the absence of peptide from the feed.

Trial design was carried out jointly by Alimetrix and AB Vista and myself; I carried out the feed formulation under the supervision of AB Vista. This experimental feed trial was carried out at Alimetrix, Finland; sample collection was carried out

according to my instructions and I assisted with DNA purification and bacterial analysis. I carried out all the analysis shown in this thesis.

A brief outline of the feed trial methodology is outlined below, full details can be found in Chapter 2 (2.19).

6.3.1. Animals and housing

Newly-hatched male Ross 508 broiler chicks (HKScan, Finland) were randomly allocated into the feeding treatments with 17 chicks per pen, 8 replicate pens and 1088 birds in total. Feed and water were available *ad libitum* at all times during the trial.

All welfare standards complied with The Finnish Act on Animal Experimentation.

6.3.2. Feed formulation

The diet was a wheat-soya based commercial-type mash feed for broiler chicks. A starter formula was used during the 3-week trial. The main feed ingredients were analysed for basic nutrients and amino acid profiles for the final formulation which can be found in Chapter 2, Table 2.3.

The test substances were carefully mixed by hand into 5 kg premixes with wheat. Each premix batch replaced 5 kg of wheat from the final formula.

6.3.3. Sample collection

The chicks were weighed on days 1, 11 and 21. Correspondingly, feed intake per pen and the feed conversion ratio (FCR) were measured for the following periods: days 1-11, days 11-21 and days 1-21. The birds were monitored on a daily basis and any dead birds or birds euthanized because of health problems were weighed. Daily mortality was recorded and FCR was calculated both corrected and uncorrected for mortality.

On day 11, two birds per pen were weighed and euthanized by cervical dislocation. The abdominal cavity was opened, and the entire ileum and the paired caeca

removed, packed in individual plastic bags, frozen immediately and stored at -20°C until analysis. On the day 21, a similar set of samples were collected, and all the remaining birds were weighed and euthanized.

Treatments were compared for significance using ANOVA, where significance was observed the Least Significant Difference test was used to determine significance between treatments.

6.3.4. Metabolite and intestinal environment analysis

The SCFAs were analysed as free acids by gas chromatography, using pivalic acid as an internal standard, as described elsewhere in detail (Holben et al., 2002). Total SCFAs, acetic, propionic, butyric, iso-butyric, 2Me-butyric, valeric, isovaleric and lactic acid were measured from the highest dose of each peptide and treatments were compared using a Kruskal-Wallis test as the data could not be normalised using typical transformations. Where significance was observed a Mann-Whitney ranked test was used to compare peptide treatments with the control.

6.3.5. Microbiome analysis

Bacterial numbers were measured using quantitative real time PCR. In this study, all results are expressed as the number of ribosomal RNA (rRNA) gene copies per 1 g of digesta. Measurements were made for the highest dose of each peptide treatment and the negative control and significance was tested using Kruskal-Wallis as the data could not be normalised using typical transformations. Where significance was observed a Mann-Whitney ranked test was used to compare peptide treatments with the control.

6.4. Results

6.4.1. Growth performance

A significant effect was observed on mean body weight gain (ANOVA, $P=0.044$). *Gallus gallus* OvoDA1 resulted in a significant dose dependant increase in mean body weight gain; it achieved a maximum 55.3 g increase when compared to the

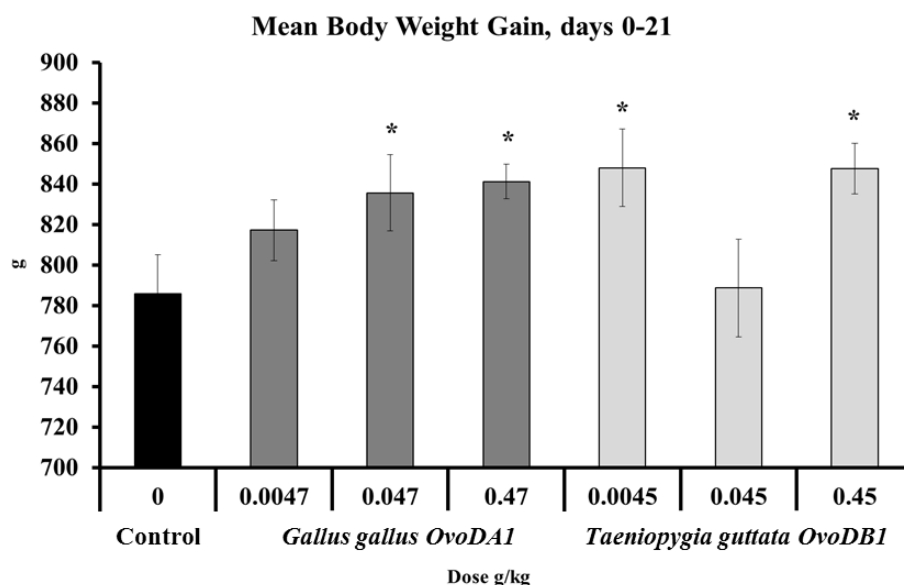


Figure 6.1: Effect of treatment on mean body weight gain from hatch to day 21 (n=136, mean \pm sem). Analysis of variance showed a significant effect on mean body weight gain (ANOVA, $P=0.044$). The significance where indicated is in comparison to with the control; $P<0.05$ (*).

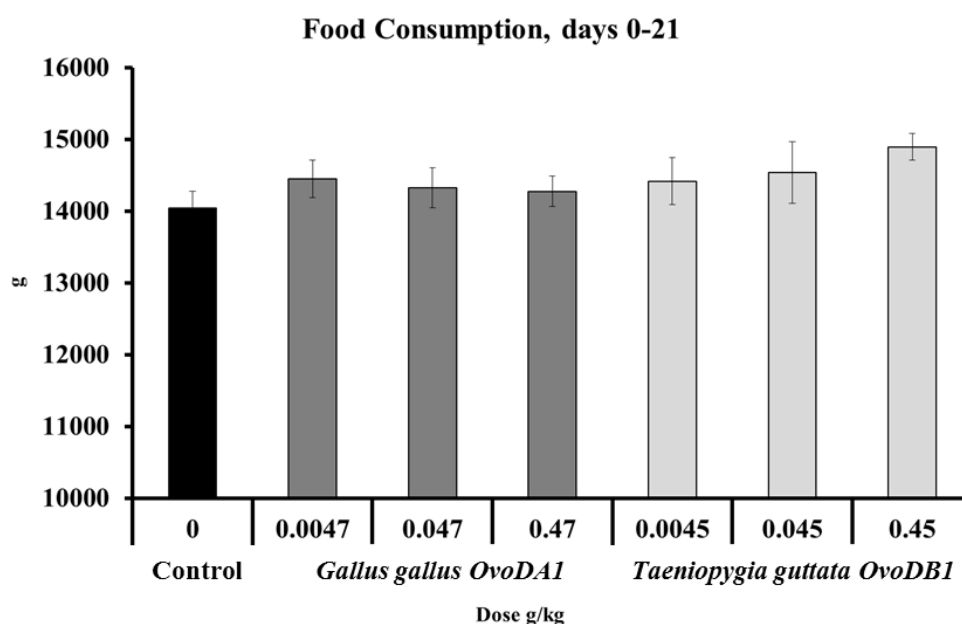


Figure 6.2: Effect of treatment on food consumption from hatch to day 21 (n=136, mean \pm sem). Analysis of variance showed there was no significant effect on food consumption (ANOVA, $P=0.540$).

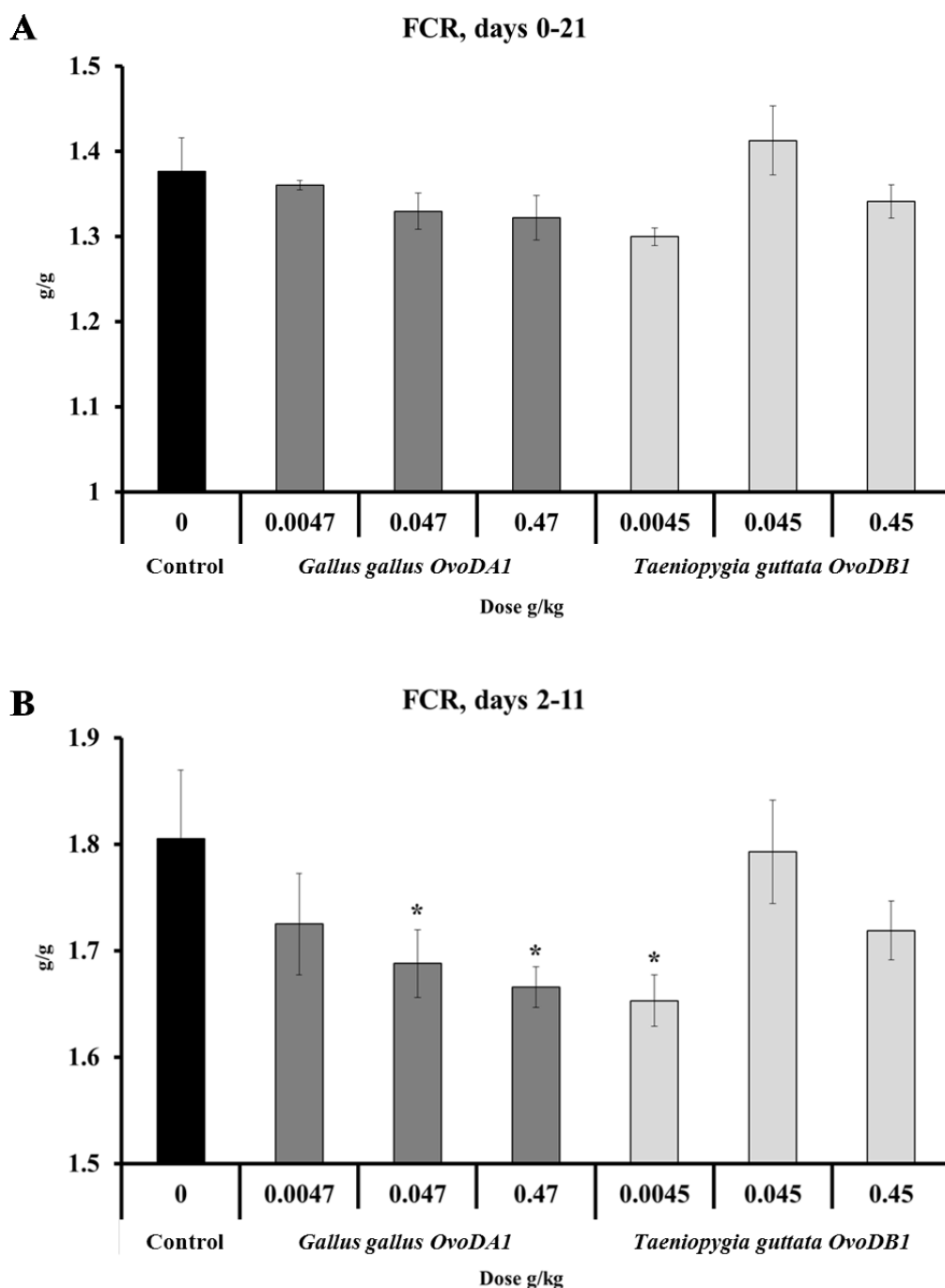


Figure 6.3: Effect of treatment on food conversion ratio (FCR) from hatch to day 21 (A) and day 2-11 (B) (n=136, mean \pm sem). Analysis of variance showed no significant effect on FCR over the course of the whole experiment, days 0-21 (ANOVA, $P=0.071$). However a significant effect on FCR was observed from day 2-11 (ANOVA, $P=0.029$). The significance where indicated is in comparison to the control; $P<0.05$ (*).

control over the total 21 day duration of the experiment ($P < 0.05$) (Figure 6.1). Inclusion of *Taeniopygia guttata* OvoDB1 at the lowest (0.0045 g/kg) and highest dose (0.45 g/kg) in the feed also resulted in a significant increase in body weight when compared with the control resulting in a maximal increase of 62.1 g at 21 days ($P < 0.05$). However no increase in body weight was observed at the middle dose (0.045 g/kg). The increase in body weight was not due to an increase in feed consumption as this did not significantly differ throughout the experiment (ANOVA, $P = 0.540$) (Figure 6.2).

Feed conversion ratio (FCR) did not significantly differ during the course of the experiment, days 0-21 (ANOVA, $P = 0.071$) (Figure 6.3A) but it approached significance, however a significant decrease in FCR was observed during the earlier growth phase of the experiment, days 2-11 (ANOVA, $P = 0.029$) (Figure 6.3B).

6.4.2. Environmental analysis

6.4.2.1. Ileum

No significant difference in total SCFAs was observed at day 11 in the ileum ($P = 0.46$) (Figure 6.4A). There was also no significant difference in acetic acid concentration ($P = 0.869$) (Figure 6.4B) or lactic acid ($P = 0.452$) (Figure 6.4C). All other parameters measured were too low to be reliably tested for significance at either day 11 or day 21.

There was also no significant effect on total SCFAs ($P = 0.946$) (Figure 6.5A), acetic acid ($P = 0.966$) (Figure 6.5B) or lactic acid ($P = 0.877$) (Figure 6.5C) at day 21.

6.4.2.2. Caeca

At day 11 there was no significant effect of total SCFAs, however the effect did verge on significance ($P = 0.056$) (Figure 6.6A). There was also no significant effect on lactic acid concentration ($P = 0.806$) (Figure 6.6B), butyric acid ($P = 0.234$) (Figure 6.6C), or propionic acid concentration ($P = 0.081$) (Figure 6.7A). However a significant effect was observed on the concentration of acetic acid in the caeca at day

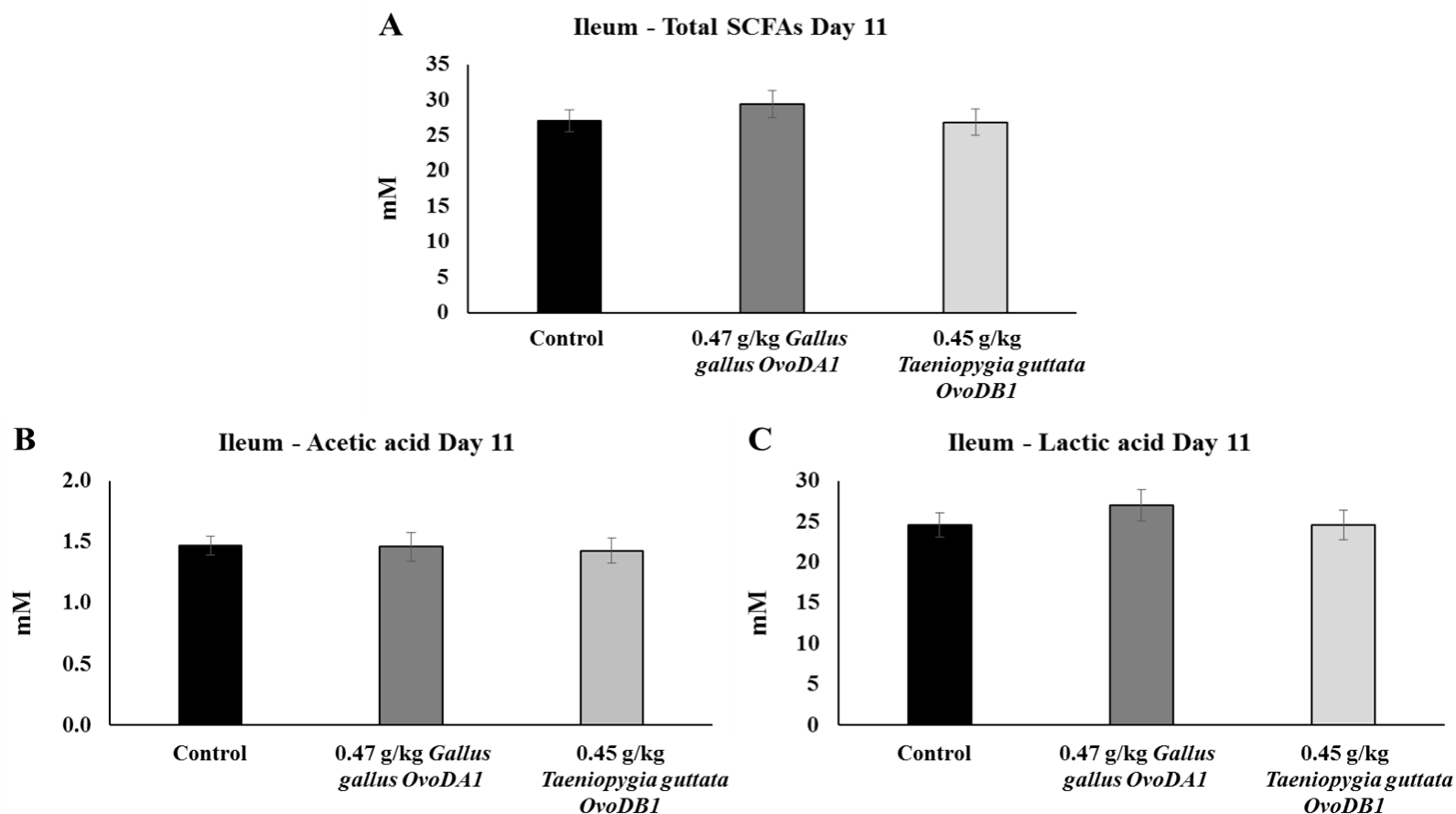


Figure 6.4: Effect of treatment on total short chain fatty acids, acetic acid and lactic acid in broiler ileum digesta at day 11 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on total short chain fatty acids ($P=0.46$) (A), acetic acid ($P=0.869$) (B) or lactic acid ($P=0.452$) (C).

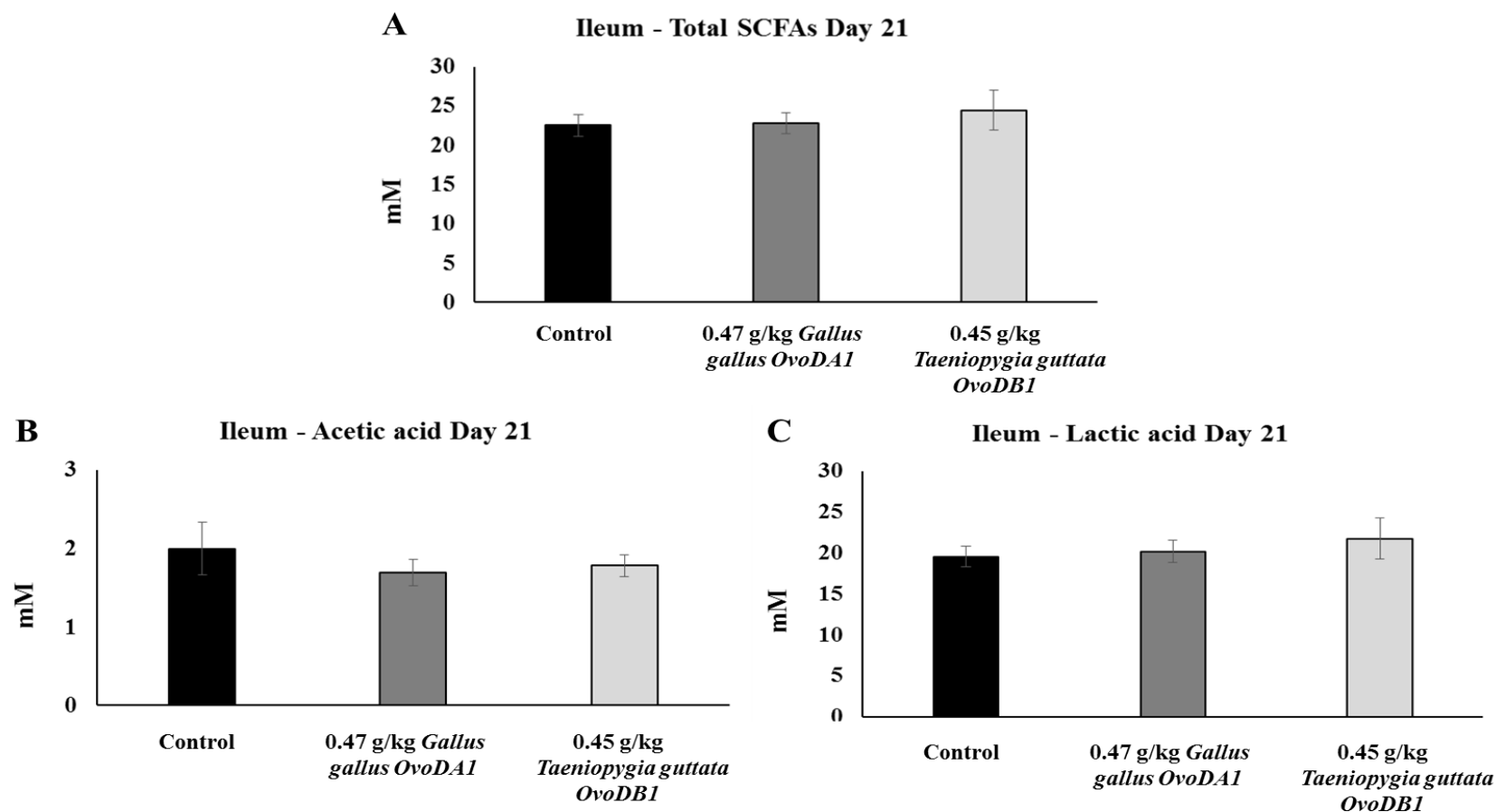


Figure 6.5: Effect of treatment on total short chain fatty acids, acetic acid and lactic acid in broiler ileum digesta at day 21 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on total short chain fatty acids ($P=0.946$) (A), acetic acid ($P=0.966$) (B) or lactic acid ($P=0.877$) (C).

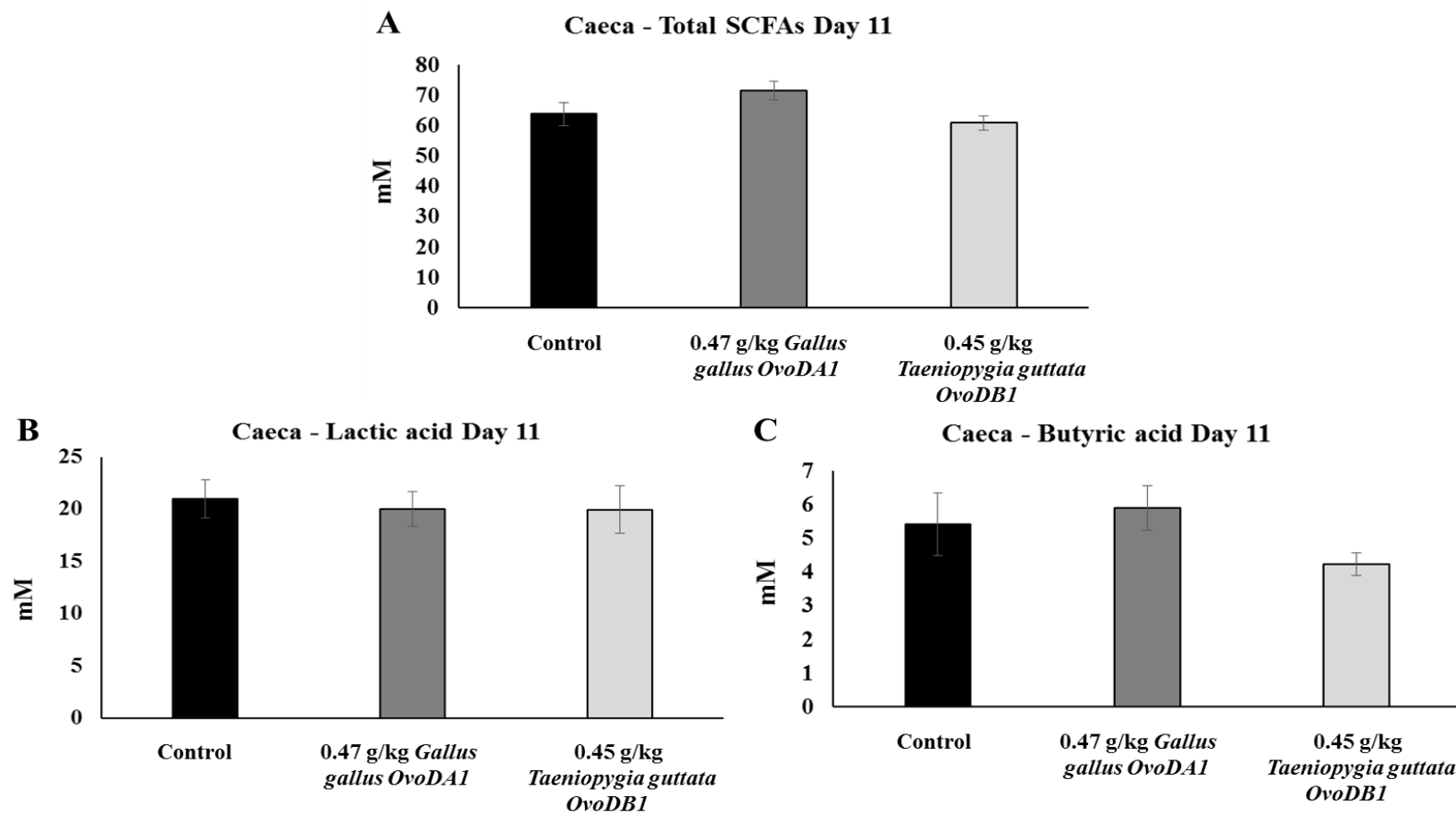


Figure 6.6: Effect of treatment on total short chain fatty acids, acetic acid and lactic acid in broiler caecal digesta at day 11 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on total short chain fatty acids ($P=0.056$) (A), lactic acid ($P=0.806$) (B) or butyric acid ($P=0.234$) (C).

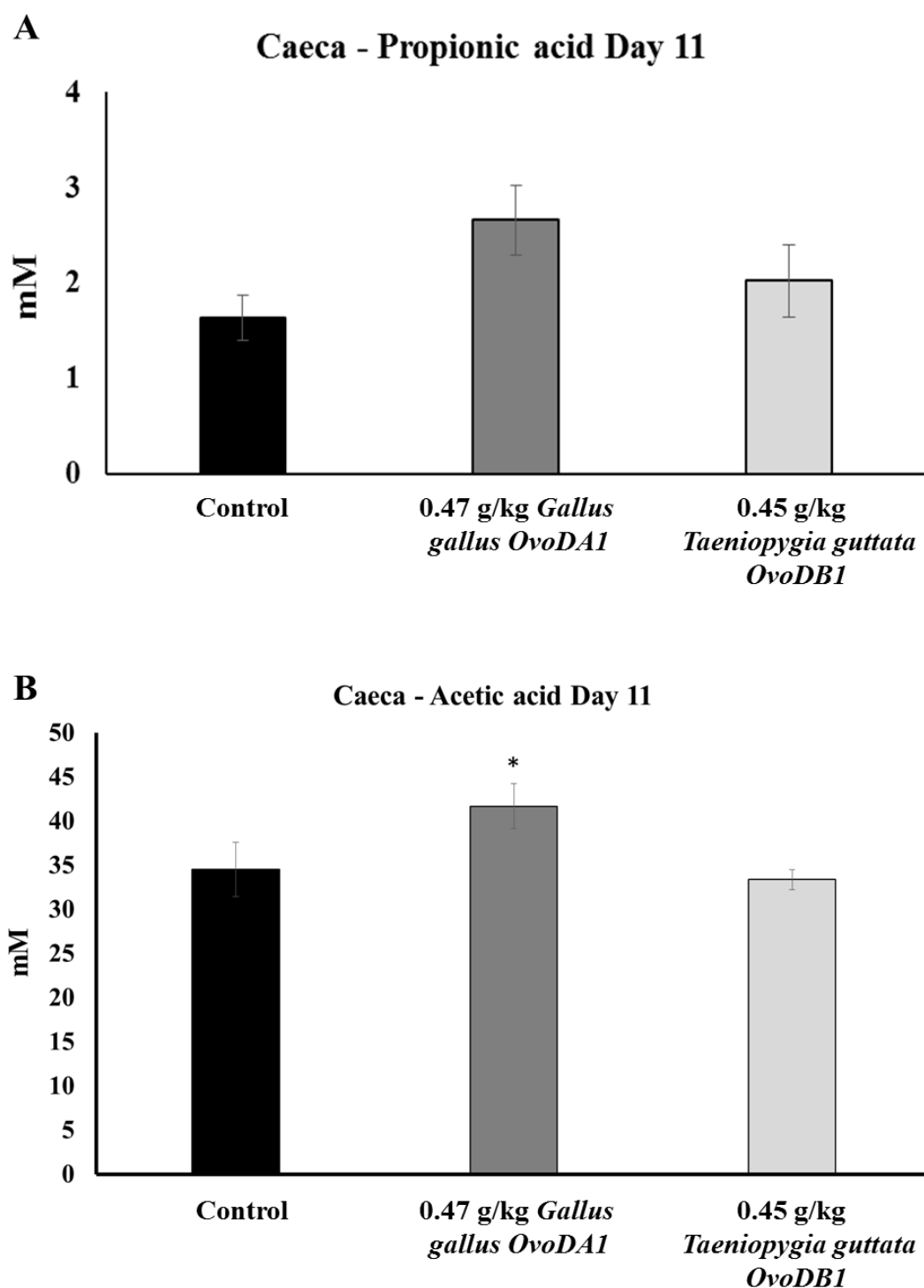


Figure 6.7: Effect of treatment on total short chain fatty acids, acetic acid and lactic acid in broiler caecal digesta at day 11 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on propionic acid ($P=0.081$) (A), however a significant effect was observed for acetic acid ($P=0.036$). The significance where indicated is in comparison to with the control; $P<0.05$ (*).

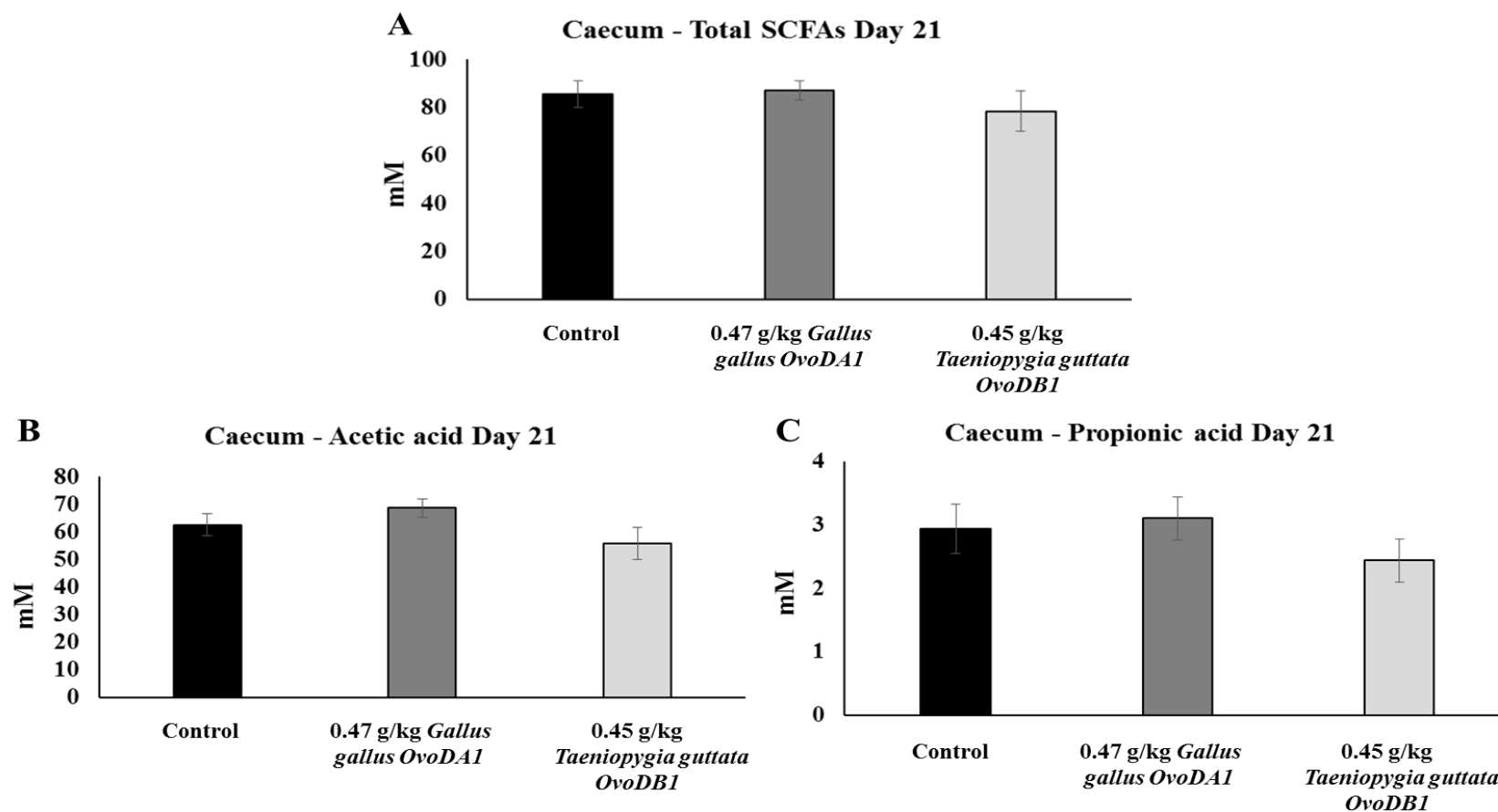


Figure 6.8: Effect of treatment on total short chain fatty acids, acetic acid and propionic acid in broiler caecal digesta at day 21 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on total short chain fatty acids ($P=0.421$) (A), acetic acid ($P=0.193$) (B) or propionic acid ($P=0.312$) (C).

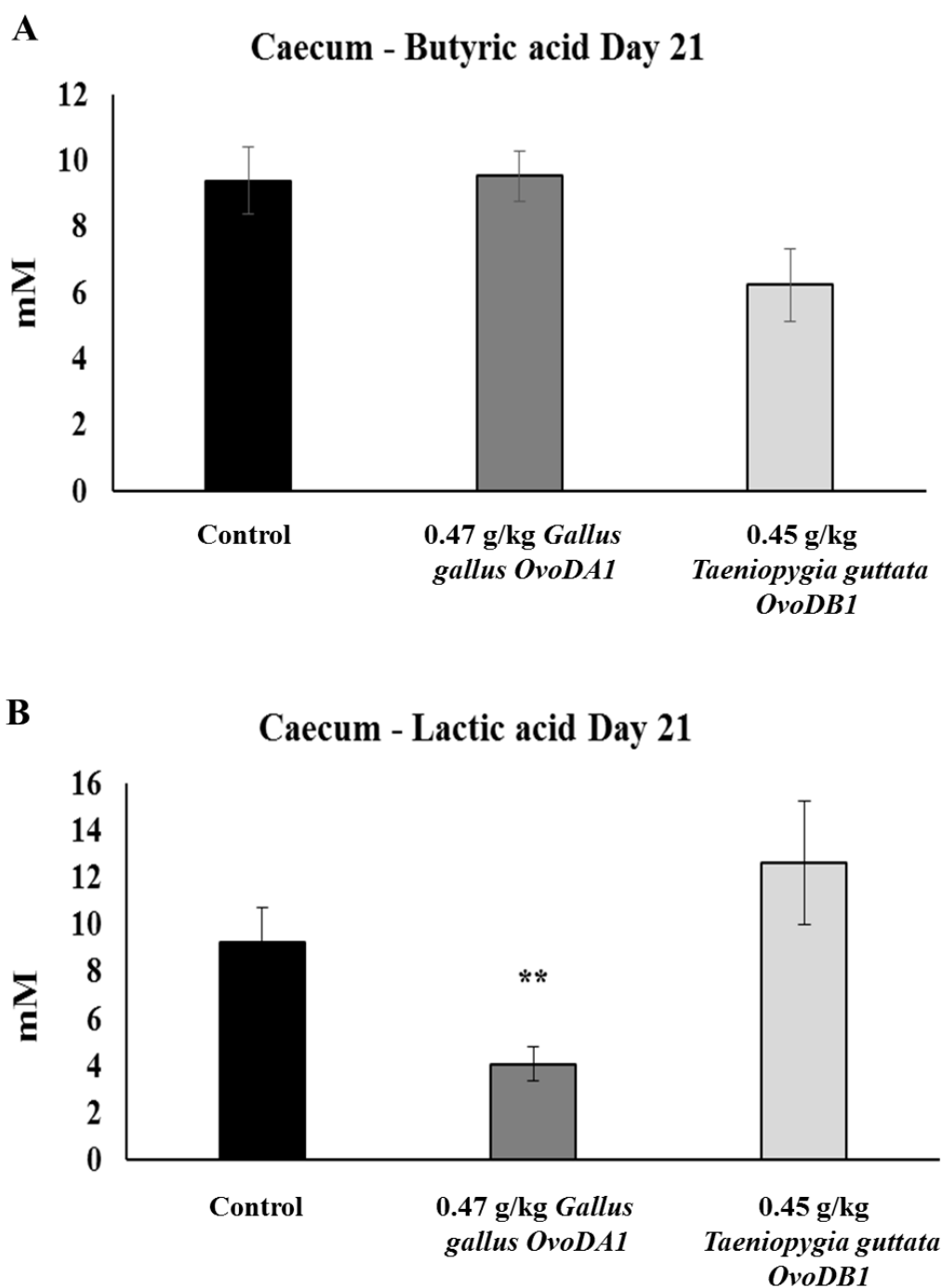


Figure 6.9: Effect of treatment on butyric acid and lactic acid in broiler caecal digesta at day 21 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on butyric acid ($P=0.076$) (A), however a significant effect was observed for lactic acid ($P=0.006$). The significance where indicated is in comparison to with the control; $P<0.01$ (**).

11 ($P=0.036$) (Figure 6.7B). A Mann-Whitney test showed that there was a significant increase in acetic acid when *Gallus gallus* OvoDA1 was included in the feed ($P=0.042$), however no significant effect was observed with *Taeniopygia guttata* OvoDB1 compared with the control ($P=0.983$). As with day 11 SCFAs there was no significant effect on total SCFAs at day 21 in the caeca ($P=0.421$) (Figure 6.8A). There was also no significant effect observed on acetic acid concentration ($P=0.193$) (Figure 6.8B) or propionic acid concentration ($P=0.312$) (Figure 6.8C). Changes in the level of butyric acid in the caeca at day 21 approached significance ($P=0.076$) (Figure 6.9A). When treatments were individually compared with the control there was no significant effect of *Gallus gallus* OvoDA1 ($P=0.751$) but again there was a trend for a reduction in butyric acid after *Taeniopygia guttata* OvoDB1 treatment ($P=0.076$). There was a highly significant effect on lactic acid concentration at day 21 in the caeca ($P=0.006$) (Figure 6.9B). When treatments were analysed individually there was no significant effect of *Taeniopygia guttata* OvoDB1 treatment ($P=0.471$) but there was a significant reduction in lactic acid concentration when *Gallus gallus* OvoDA1 was included in the diet ($P=0.012$).

6.4.3. Microbiome analysis

6.4.3.1. Ileum

Microbiome analysis of day 11 ileum digesta showed no significance in the total number of bacteria ($P=0.49$) (Figure 6.10A) between treatments. Specifically there was no significant change in the number of total *Lactobacillus* ($P=0.43$) (Figure 6.10B) or *Enterococcus* species ($P=0.847$) (Figure 6.10C). There was also no significant change in *Lactobacillus reuteri* ($P=0.778$) (Figure 6.11A), *Clostridium perfringens* ($P=0.715$) (Figure 6.11B), *Escherichia coli* ($P=0.759$) (Figure 6.11C) or Streptococci ($P=0.097$) (Figure 6.11D). *Eimeria* levels in the ileum were measured but were undetectable in almost all samples.

Day 21 ileal digesta results indicated no significant changes in total numbers of bacteria ($P=0.544$) (Figure 6.12A). There were also no significant differences in

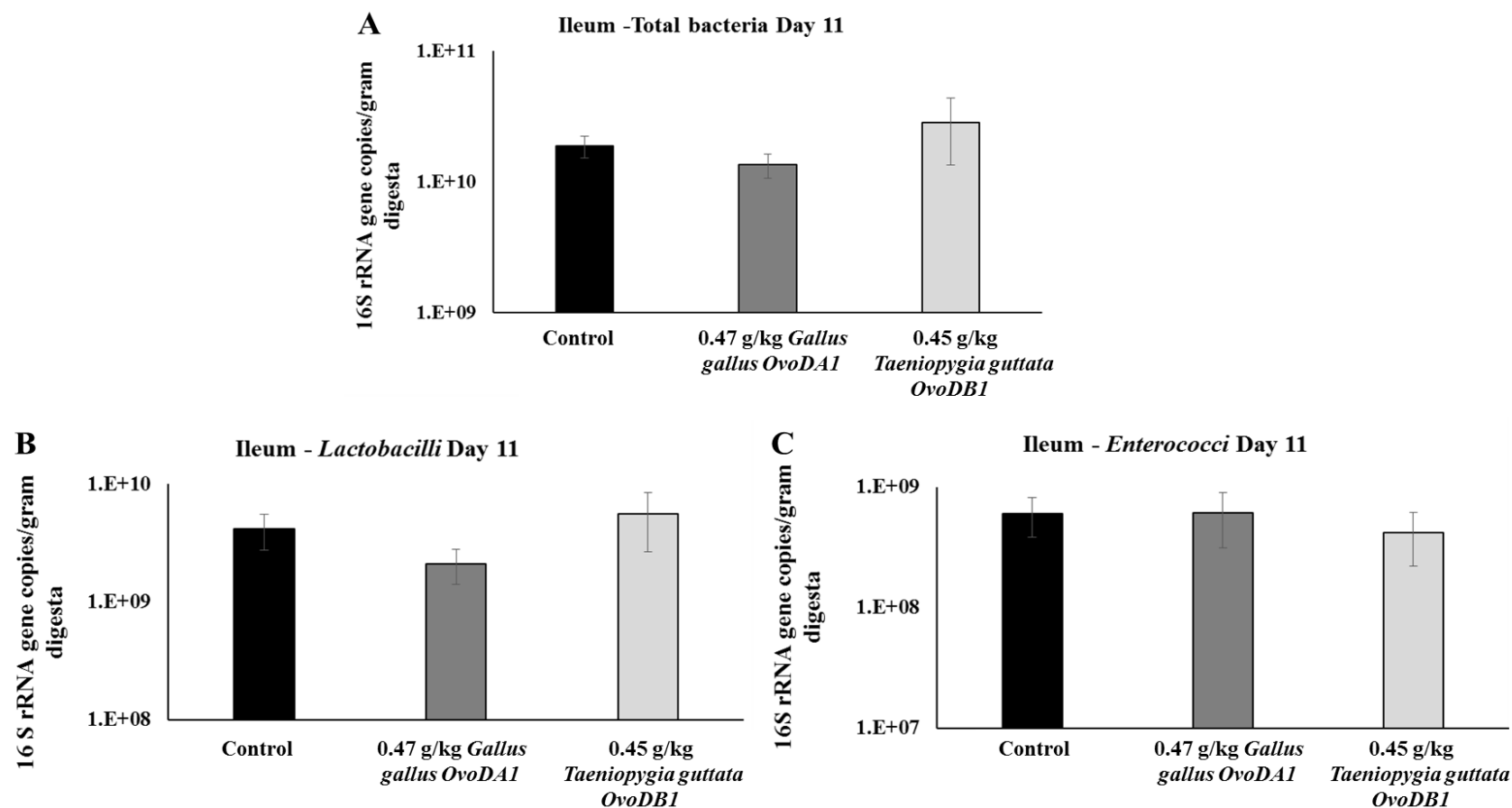


Figure 6.10: Effect of treatment on total bacteria, *Lactobacillus* and *Enterococcus* in broiler ileum digesta at day 11 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on total bacteria (P=0.49) (A), *Lactobacillus* species (P=0.43) (B) or *Enterococcus* numbers (P=0.847) (C).

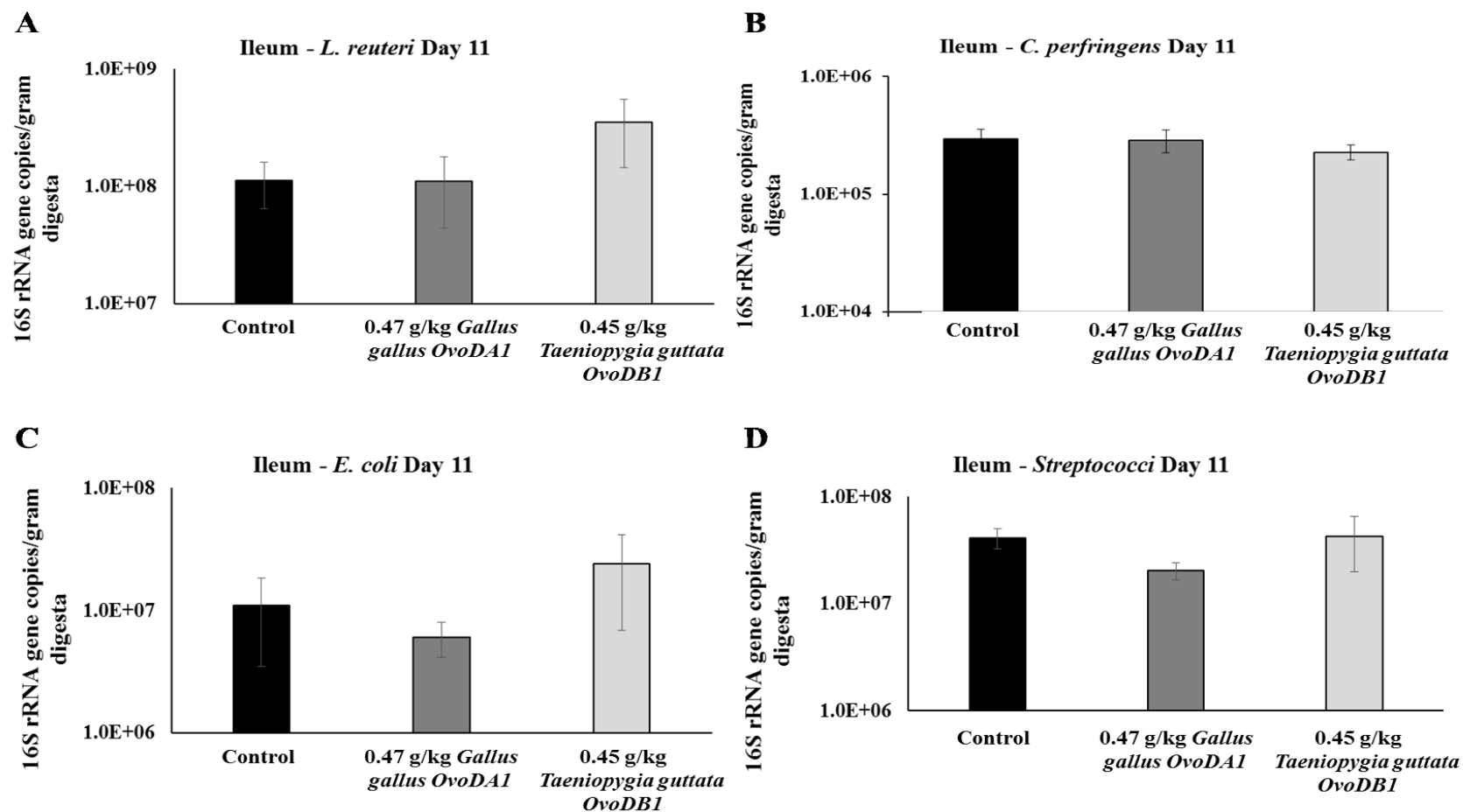


Figure 6.11: Effect of treatment on *Lactobacillus reuteri*, *Clostridium perfringens*, *E. coli* and *Streptococcus* in broiler ileum digesta at day 11 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on *Lactobacillus reuteri* (P=0.778) (A), *C. perfringens* (P=0.715) (B), *E. coli* (P=0.759) (C) or *Streptococcus* numbers (P=0.097) (D).

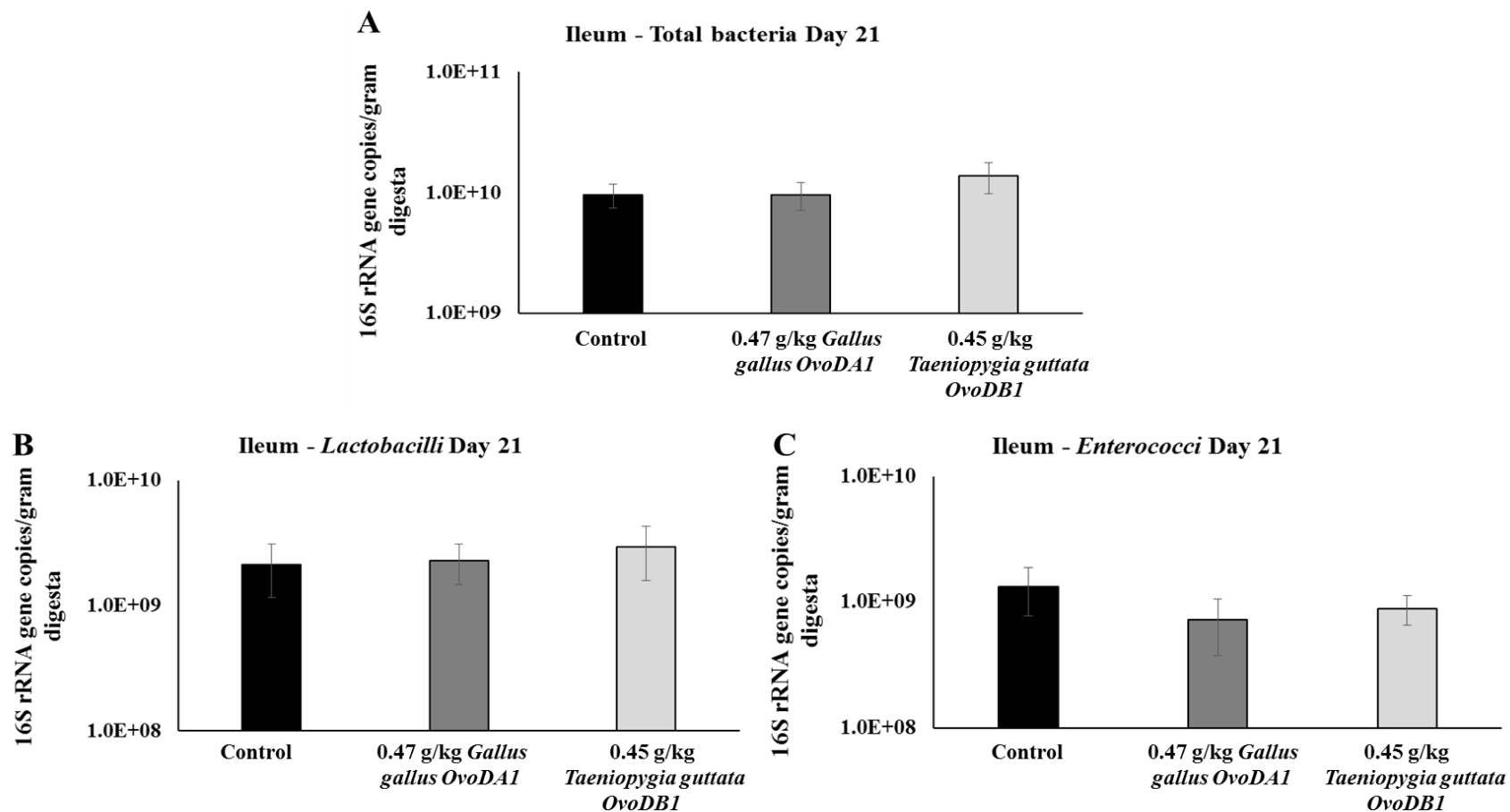


Figure 6.12: Effect of treatment on total bacteria, *Lactobacillus* and *Enterococcus* in broiler ileum digesta at day 21 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on total bacteria (P=0.544) (A), *Lactobacillus* species (P=0.803) (B) or *Enterococcus* numbers (P=0.221) (C).

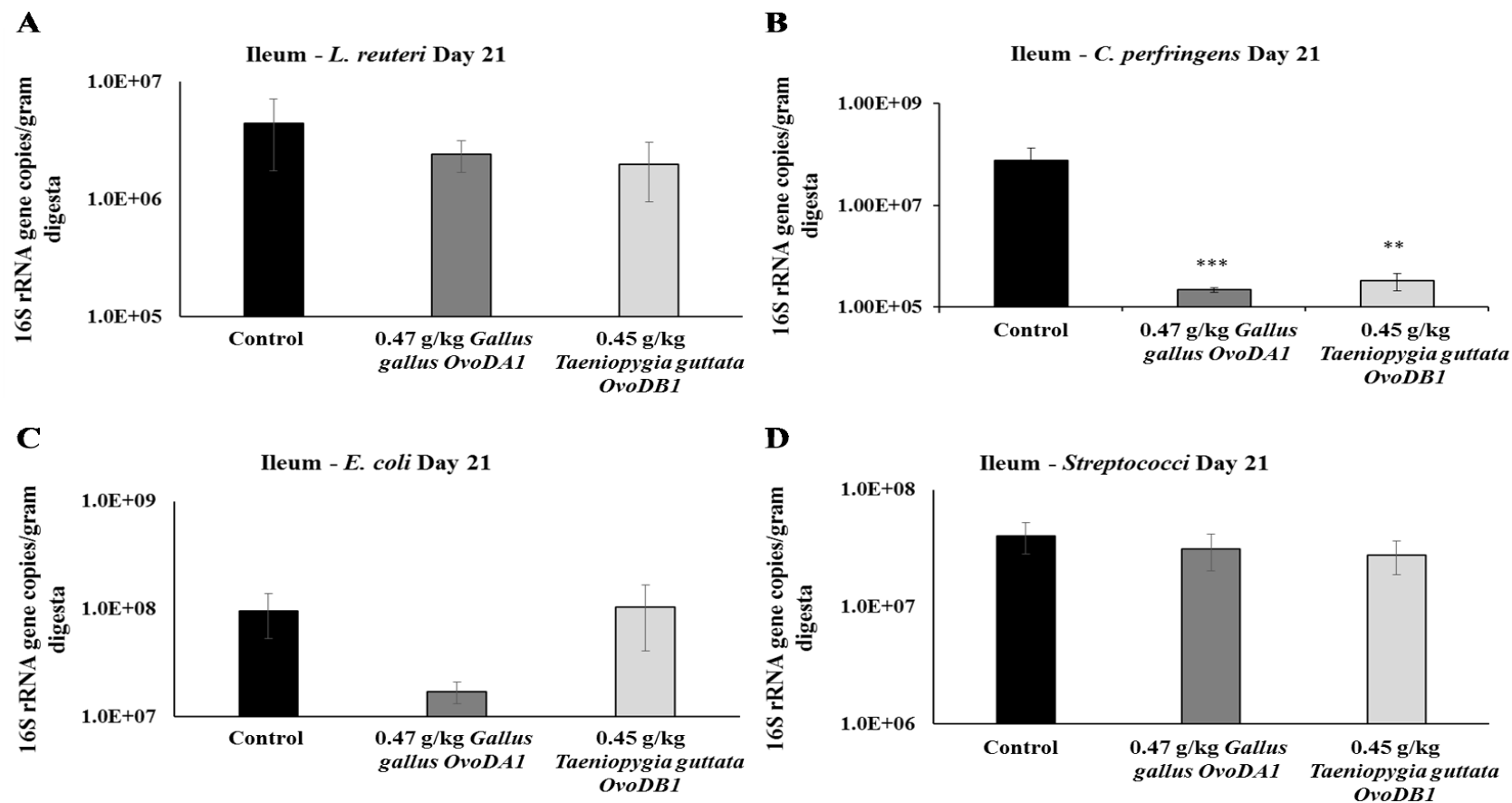


Figure 6.13: Effect of treatment on *Lactobacillus reuteri*, *Clostridium perfringens*, *E. coli* and *Streptococcus* in broiler ileum digesta at day 21 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on *Lactobacillus reuteri* (P=0.434) (A) however there was a highly significant effect on *C. perfringens* levels (P=0.002) (B). There was also no significant difference in *E. coli* (P=0.202) (C) or *Streptococcus* numbers (P=0.332) (D). The significance where indicated is in comparison to with the control; P<0.01 (**) and P<0.001 (***).

Lactobacillus species ($P=0.803$) (Figure 6.12B), *Enterococcus* ($P=0.221$) (Figure 6.12C) or *Lactobacillus reuteri* ($P=0.434$) (Figure 6.13A). A highly significant change was observed in the level of *Clostridium perfringens* in the ileum at day 11 ($P=0.002$) (Figure 13B) that was still significant when a Bonferroni correction was applied. Both treatment with 0.47 g/kg *Gallus gallus* OvoDA1 and 0.45 g/kg *Taeniopygia guttata* OvoDB1 resulted in a highly significant reduction in *C. perfringens* ($P=0.0016$ and $P=0.006$ respectively). No significant changes were observed in *E. coli* ($P=0.202$) (Figure 6.13C) however from the graph there did appear to be some large inter group differences so individual treatments were assessed and the effect of *Gallus gallus* OvoDA1 approached significance ($P=0.062$). No significant changes were observed for *Streptococcus* ($P=0.332$) (Figure 6.13D). As in the day 11 ileal digesta *Eimeria* was below the level of detection of this assay.

6.4.3.2. Caeca

Analysis of day 11 caecal contents did not show a significant difference in the total number of bacteria ($P=0.599$) (Figure 6.14A). There was also no significant change in *Lactobacillus* ($P=0.255$) (Figure 6.14B), *Bifidobacterium* ($P=0.249$) (Figure 6.14C) or *Coriobacteriaceae* species ($P=0.200$) (Figure 6.14D). However there was a significant effect on Clostridial cluster IV organisms in the caeca ($P=0.044$) (Figure 6.15A). Specifically when treated with *Gallus gallus* OvoDA1 there was a significant increase in Clostridial cluster IV organisms ($P=0.029$), however no changes were observed with *Taeniopygia guttata* OvoDB1 treatment ($P=0.429$). Although not significant there was also a trend for an increase in Clostridial cluster XIVa ($P=0.076$) (Figure 6.15B). No changes were observed in the caeca at day 11 for *E. coli* ($P=0.129$). Both *Eimeria* and *Megamonas hypermegale* were too low to be measured accurately in this assay.

The total number of bacteria did not significantly differ at day 21 in the caeca ($P=0.324$) (Figure 6.16A). There was also no change in total *Lactobacillus* species ($P=0.137$) (Figure 6.16B). There was a highly significant effect on *Bifidobacterium*

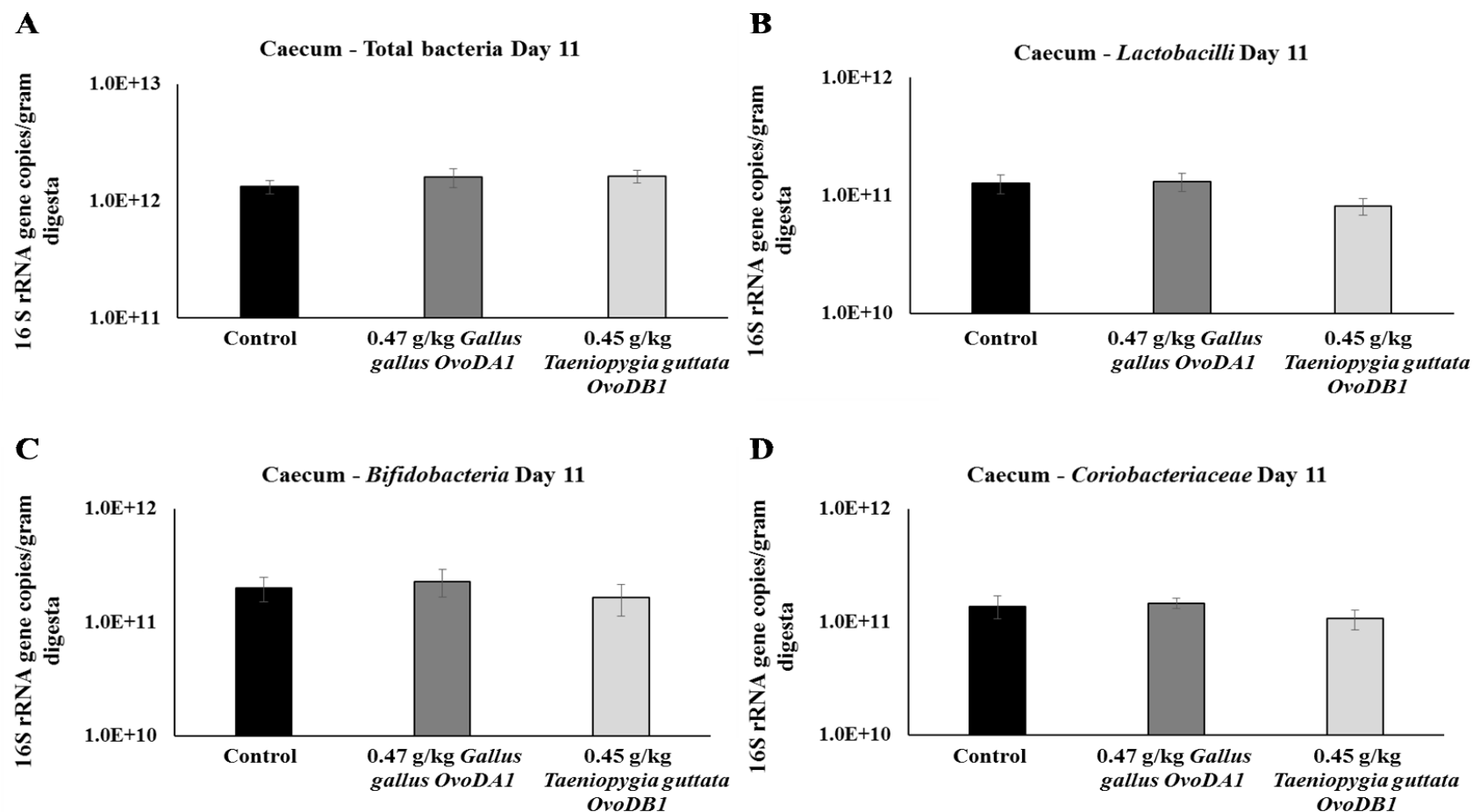


Figure 6.14: Effect of treatment on total bacteria, *Lactobacillus*, *Bifidobacteria* and *Coriobacteriaceae* in broiler caecal digesta at day 11 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on total bacteria (P=0.599) (A), *Lactobacillus* species (P=0.255) (B), *Bifidobacteria* (P=0.249) (C) or *Coriobacteriaceae* numbers (P=0.200) (D).

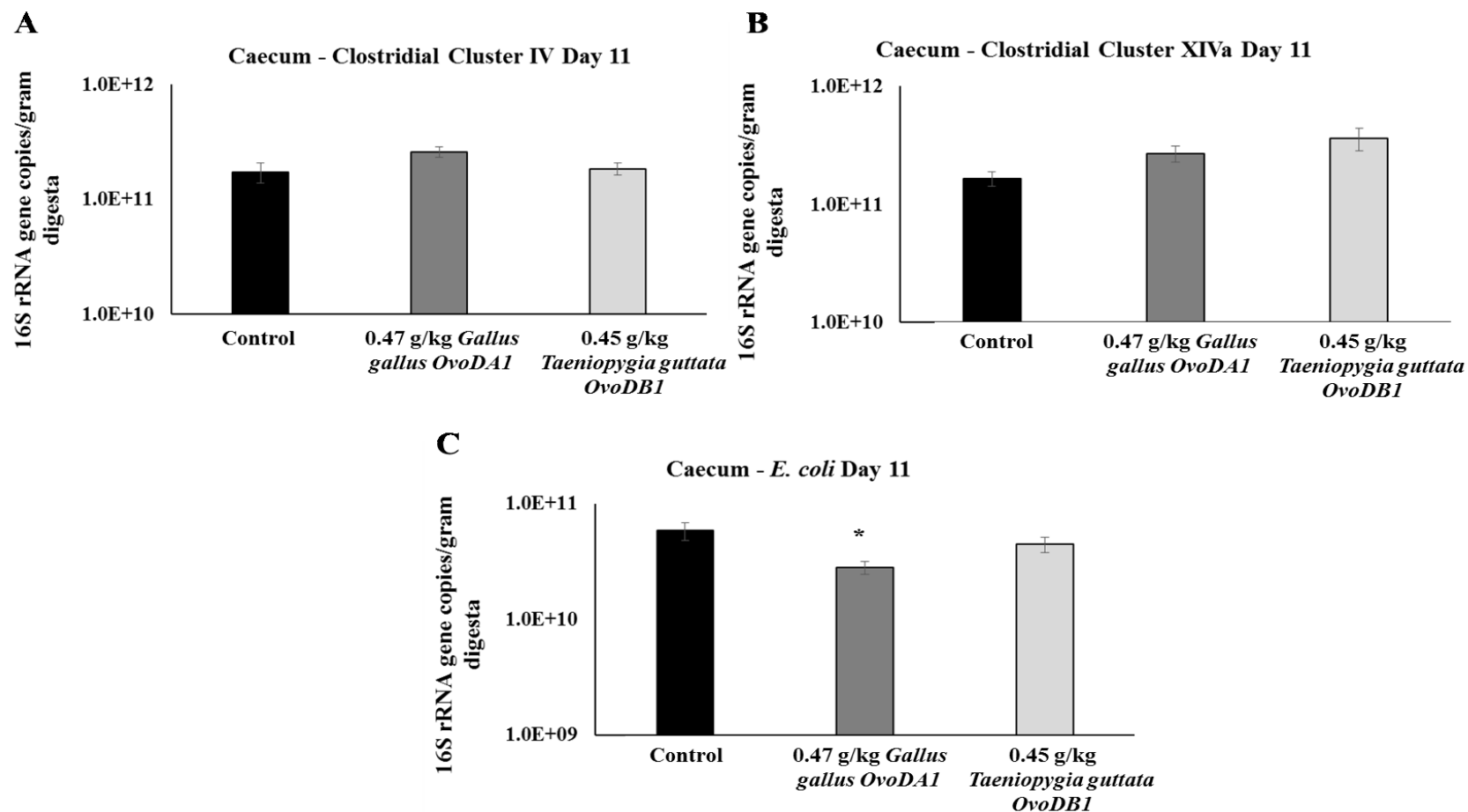


Figure 6.15: Effect of treatment on Clostridial cluster IV, Clostridial cluster XIVa and *E. coli* in broiler caecal digesta at day 11 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed a significant effect on Clostridial cluster IV (P=0.044) (A) and the effect on Clostridial cluster XIVa verged on significance (P=0.076) (B). There was no significant effect on *E. coli* (P=0.129) (C).

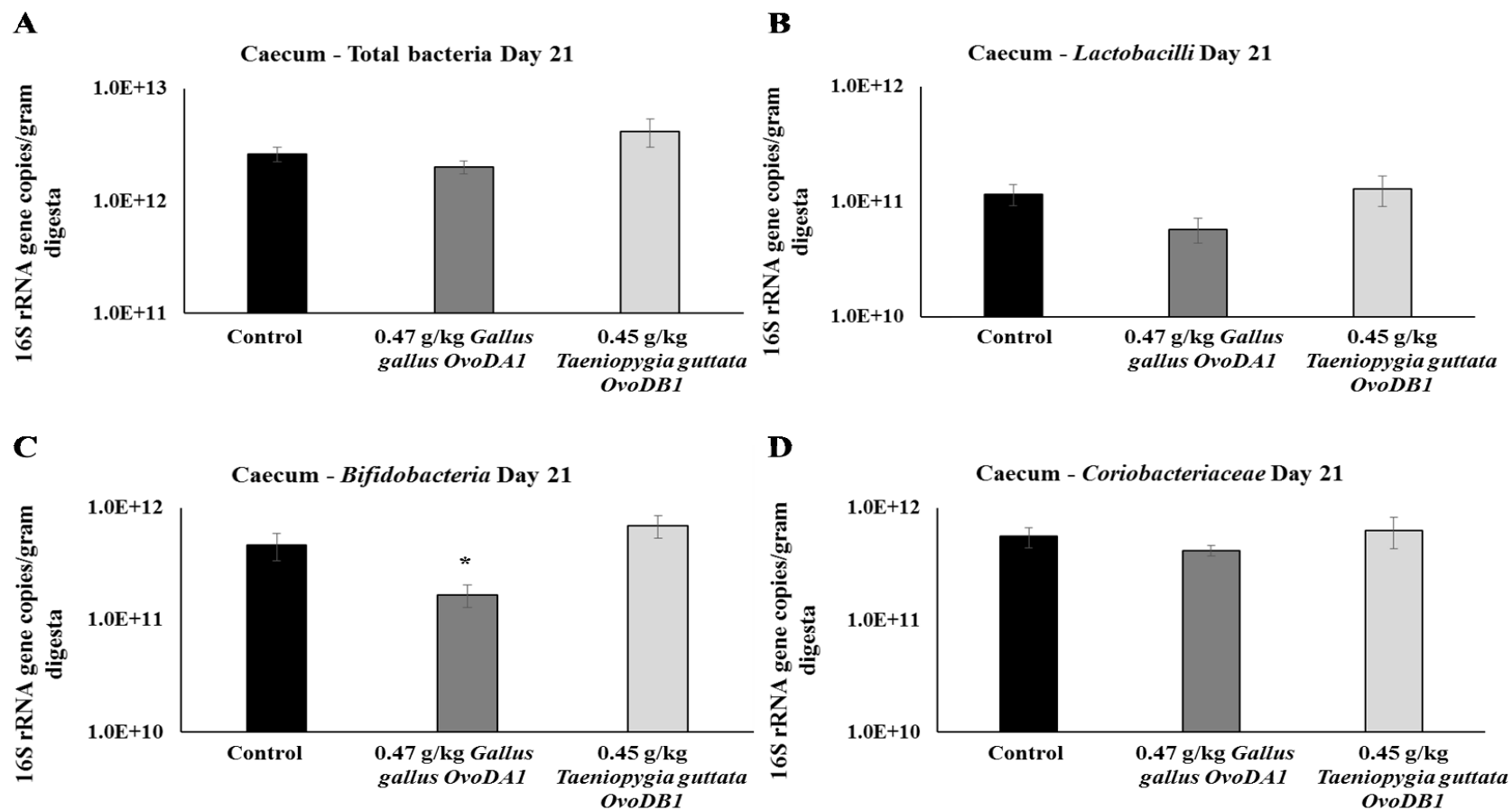


Figure 6.16: Effect of treatment on total bacteria, *Lactobacillus*, *Bifidobacteria* and *Coriobacteriaceae* in broiler caecal digesta at day 21 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on total bacteria (P=0.324) (A) or *Lactobacillus* species (P=0.137) (B). There was a highly significant effect on *Bifidobacteria* numbers in the caeca (P=0.001) (C) at day 21. No significant effect was observed on *Coriobacteriaceae* numbers (P=0.893) (D).

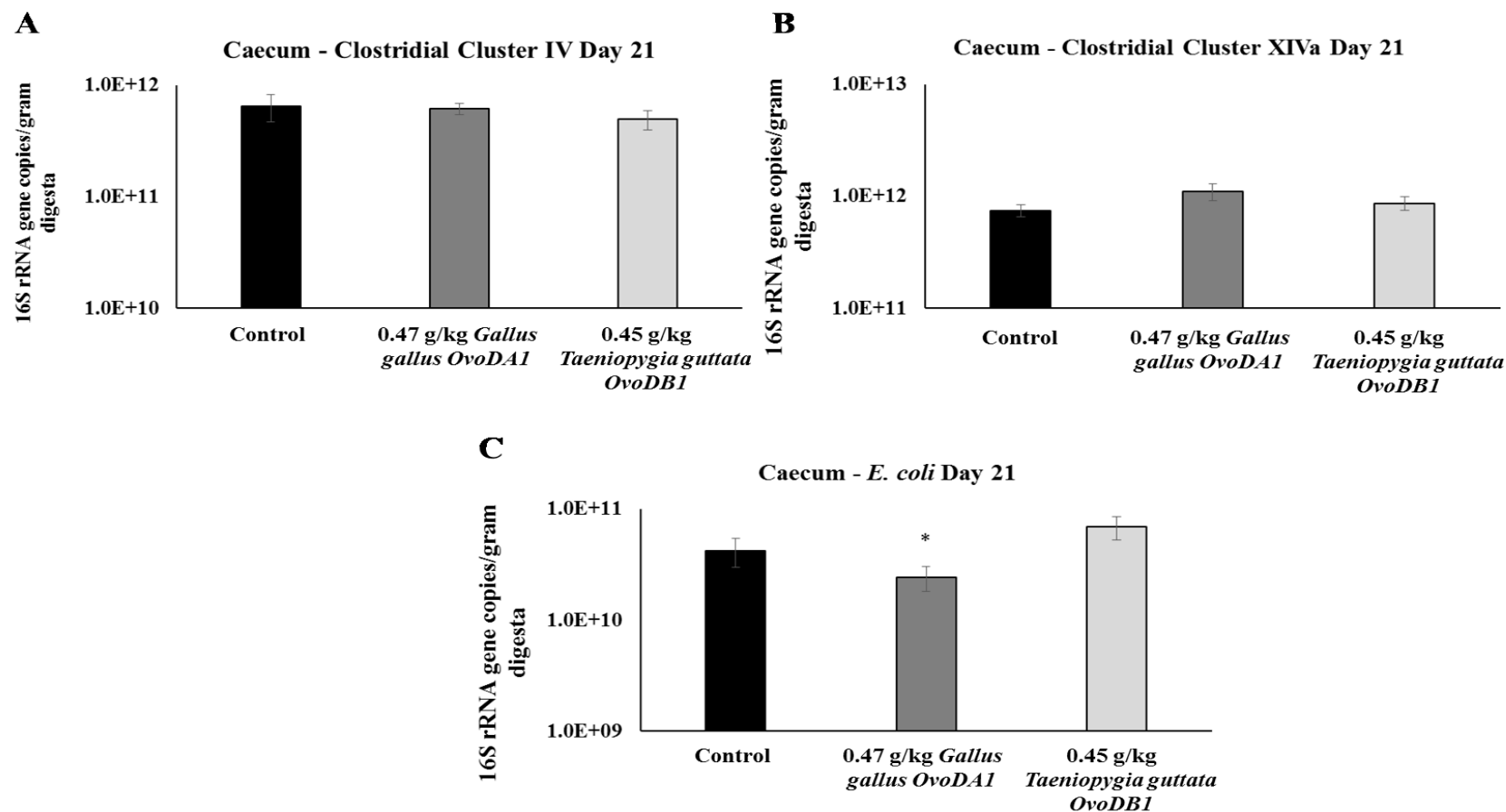


Figure 6.17: Effect of treatment on Clostridial cluster IV, Clostridial cluster XIVa and *E. coli* in broiler caecal digesta at day 21 (n=16, mean \pm sem). Analysis by a Kruskal-Wallis test showed no significant effect on Clostridial cluster IV ($P=0.395$) (A) or Clostridial cluster XIVa ($P=0.455$) (B). However there was a significant effect on *E.coli* numbers in the caeca at day 21 ($P=0.013$) (C). The significance where indicated is in comparison to with the control; $P<0.05$ (*).

levels in the caeca at day 21 ($P=0.001$) (Figure 6.16C) which was still significant when a Bonferroni correction was applied. When treatments were compared with the control *Gallus gallus* OvoDA1 resulted in a significant reduction in *Bifidobacterium* ($P=0.027$) whereas the effect of *Taeniopygia guttata* OvoDB1 was not significant ($P=0.189$). However the effect of *Gallus gallus* OvoDA1 versus the control did not explain the large significance observed in the data and when the two peptides were compared with each other a highly significant difference between treatments was observed ($P=0.0004$). At day 21 no significant changes were observed in *Coriobacteriaceae* ($P=0.893$) (Figure 6.16D), Clostridial cluster IV ($P=0.395$) (Figure 6.17A) or Clostridial cluster XIVa ($P=0.455$) (Figure 6.17B). There was a significant effect on *E. coli* numbers ($P=0.013$) (Figure 6.17C); compared with the control *Gallus gallus* OvoDA1 resulted in a significant reduction ($P=0.046$) whereas there was no difference after *Taeniopygia guttata* OvoDB1 treatment ($P=0.141$). *Megamonas hypermegale* and *Eimeria* were still below the limits of detection in the caeca at day 21.

6.5. Discussion

Ovodefensin peptide treatment had a significant effect on mean body weight gain over the duration of the experiment (Figure 6.1). *Gallus gallus* OvoDA1 resulted in a dose dependant increase in mean body weight; for inclusion at both 0.047 and 0.47 g/kg the increase was significant when compared to the un-supplemented diet. *Taeniopygia guttata* OvoDB1 feed supplementation also resulted in an increase in body weight when compared with the control at 0.0045 and 0.45 g/kg. The increase in body weight was not due to an increase in feed consumption as this did not alter over the course of the experiment (Figure 6.2). Strangely there was no significant increase in body weight with the middle dose (0.045 g/kg) of *Taeniopygia Guttata* OvoDB1 despite the fact that there was an increase with the lowest dose as well as a reduction in FCR. This may suggest some benefit to examining lower doses of ovodefensin peptide inclusion; perhaps at lower doses the peptide results in less of an immunomodulatory response. As this response requires expenditure of energy (Whittow, 1999) effective dosing of peptide may require finding an appropriate

balance between energy spent on altering the gut microflora and that which is gained through this microbiome alteration. However it should be noted that despite there being a large amount of replication within the experiment this is the first time these peptides have been tested in this way and a repeat of this work will need to be carried out to confirm that this is a true observation. Currently if these results are corrected using the Bonferroni method most would fall below the criteria of significance but the results give an indication of which parameters may be important to assess in the future. That said, even with that stringent correction there are some cases in these experiments where significance is achieved. In the final chapter recommendations for future feed trials and further analysis on the current samples in order to determine the true effects of these peptides will be discussed.

Observations from the analysis of both the environmental profile of the gastrointestinal tract e.g. pH and metabolites as well as the microbiome showed beneficial effects, particularly in the caeca. At day 11 peptide inclusion resulted in a significant increase in butyrate producing Clostridial cluster IV (Figure 6.15A) and an increase in Clostridial cluster XIVa that approached significance (Figure 6.15B) ($P=0.076$). Butyrate is produced through bacterial fermentation of undigested carbohydrates (Pryde et al., 2002) and it has been suggested that it can induce endogenous defensin expression (Sunkara et al., 2011) which may in turn promote beneficial modulation of the gastrointestinal microbiome. In support of this observation there was a strong trend (Figure 6.9A) in the increase of butyrate levels in the caeca by day 21. It was not possible in this thesis to measure endogenous expression of defensins due to time but tissue samples were collected for this purpose and it would be interesting to see if stimulating butyrate production *in vivo* results in an increase in AvBD9 and AvBD14 as previously described when butyrate was administered directly to the bird through the feed (Sunkara et al., 2011). By day 21 there was also a highly significant decrease in *Bifidobacterium* in the caeca (Figure 6.16C). Analysing treatments individually demonstrated that only *Gallus gallus* *OvoDAI* produced a significant effect when compared with the control. Interestingly the most statistically significant difference in *Bifidobacterium* was observed when

comparing peptide treatments with each other possibly suggesting they have opposing effects on this organism. *Bifidobacterium* metabolise simple carbohydrates and can help utilise those carbohydrates which have escaped host digestion (Willey et al., 2008). *Bifidobacterium* is generally considered a beneficial commensal of the GIT and is often used as a probiotic as it is associated with pathogen exclusion, particularly competitive exclusion of gram negative organisms, and has also been implicated in maintenance of the intestinal barrier (Griffiths et al., 2004). However, although these organisms are generally considered beneficial they metabolise fairly simple carbohydrates and therefore high numbers in the caeca can be an indication of poor nutrient absorption further up in the GIT (Rinttila and Apajalahti, 2013). Therefore the highly significant reduction of these organisms in the caeca suggest that nutrient absorption is improved in the peptide treated birds meaning that less unabsorbed sugars are reaching the caeca. At day 21 there was also a highly significant effect on lactic acid concentration in the caeca (Figure 6.9B), *Gallus gallus OvoDA1* in particular resulted in a large reduction in lactic acid; as lactic acid is a major end product of *Bifidobacteria* metabolism (Willey et al., 2008) this correlates nicely with the reduction in numbers of this organism.

No significant changes were observed in the ileum for any of the SCFA profiles measured in this study. However as previously discussed the reduction of *Bifidobacterium* and lactic acid in the caeca is indicative of improved nutrient absorption in the higher gastrointestinal tract. It may be that these improvements could not be identified from the parameters measured or it may be that these changes were happening more proximally in the GIT, for example in the jejunum where a large amount of nutrient digestion and absorption takes place (Whittow, 1999). At day 11 there was a reduction in *Streptococcus* in the ileum which approached significance (Figure 6.11D). Although *Streptococcus* is considered a commensal organism in the gut it can be an opportunistic pathogen (Willey et al., 2008) and a reduction in this genus could therefore be considered beneficial. At day 21 no significance was detected in *E. coli* levels in the ileum when Kruskal Wallis was used to analyse the data (Figure 6.13C), however the graph indicated that there was a

strong tendency for a reduction in *E. coli* numbers when *Gallus gallus* OvoDA1 was administered. Therefore a Mann-Whitney test was used to test the significance between the negative control and *Gallus gallus* OvoDA1, this showed that the trend approached significance (Figure 6.13C). Most strikingly peptide administration had a very significant effect on the number of *Clostridium perfringens* organisms in the ileum at day 21 (Figure 6.13B) with numbers being reduced with both peptides. *Clostridium perfringens* is the causative agent of necrotic enteritis (McDevitt et al., 2006) and has been of particular concern to the poultry industry since the ban on antibiotic growth promoters in the last decade (Dahiya et al., 2006). Any reduction in this organism could have considerable benefits to the poultry industry both in terms of the health and performance of the flock and consequently welfare and profit.

The observations made in this chapter suggest that the in-feed inclusion of ovodefensin peptides could present an interesting alternative to traditional antibiotic growth promoters (AGPs). The increase in body weight observed paralleled that achieved, a maximal increase of 62.1 g at 21 days with the AGPs (Miles et al., 2006) and the changes observed in both the environmental profile and microbiome were indicative of improved nutrient absorption and a healthy microbiota. Further work will need to be carried out to determine if these observations are reproducible and whether they offer protection from disease in a challenge situation; this will be discussed in detail in the next chapter.

Chapter 7

Final discussion

7.1. Final discussion

In this final chapter the major findings of this research will be summarised and the potential implications of the work will be discussed. In particular directions for future research with a focus on commercialisation and understanding the mechanisms behind the changes observed in the *in vivo* peptide trial will be addressed.

7.1.1. General discussion

7.1.1.1. TENP

The work outlined in Chapter 3 gives new insights into the comparative biology and expression of TENP, demonstrating its conservation among avian species and describing for the first time its expression in the adult hen. The results strongly suggest that in the adult TENP plays a major role in the egg, most probably in antimicrobial innate defence. It is possible that TENP may also act locally in the oviduct providing antimicrobial protection; however, immunohistochemistry showed no TENP protein in the epithelial cells suggesting that its major role is within the egg. Chapter 3 also described in detail the transcript encoding TENP in the adult, showing that both BPI domains are expressed and revealing an alternative translational start site and frameshift event compared with the previously published sequence (Yan and Wang, 1998). In the adult TENP has the clear signature of an egg specific protein; its expression is restricted to the oviduct, diminished when the bird is out of lay and enhanced in response to gonadal steroids. This is particularly interesting because in the embryo TENP is expressed in brain and retinal tissues and at least in the brain may play a transient role in development (Yan and Wang, 1998). The dual purpose of TENP makes it an extremely interesting candidate for future research as it could provide insight into the biology of not only the egg but the chick as well.

It was decided that TENP would not be the focus of feed trials in this project due to its large size and efforts in protein production and assessment of antimicrobial activity were directed elsewhere. However since this work was completed

antimicrobial activity has been demonstrated for emu TENP (Maehashi et al., 2014) and it would be extremely interesting to see if chicken TENP also possessed antimicrobial activity. The large size and consequent multiple protease cleavage sites of TENP make it unsuitable as a feed additive; however if shown to be antimicrobial it may be useful in other applications such as carcass washing where the route of application is topical, or outside of the poultry industry in functions such as wound healing or catheter sterilisation (Easton et al., 2009). TENPs proposed mechanism of action is through LPS binding (Beamer et al., 1998); it may therefore provide a useful synergistic relationship with molecules such as the ovodefensins which are proposed to rely on electrostatic interactions as typical of β -defensins (Ganz, 2003).

7.1.1.2. The ovodefensin family

In this thesis it was shown that the ovodefensins are a structurally distinct family of positively charged β -defensins which are conserved across avian and reptilian species. Within the ovodefensin family unique cysteine spacing motifs were identified which divide this group into six sub-families which were used to propose new nomenclature. Evolutionary analysis suggests that one cysteine motif (OvoDB) has evolved independently in both birds and reptiles demonstrating unique evolution of spacing within this family as well as classical evolution of sequence. RT-qPCR analysis confirmed that at least in the avian species examined in this study, expression was restricted to the oviduct as previously documented for *Gallus gallus* *OvoDA1* (*gallin*) (Gong et al., 2010). However it was apparent that whilst the level of expression in each oviduct region varied between species it was fixed for each complement of ovodefensins within a species. This finding poses the question; what is driving the evolution of ovodefensins and specifically what might be driving expression? For example it could be hypothesised that the environment in which a given species lays its eggs affects which regions of the egg most require antibacterial protection, and ovodefensin expression would be up-regulated accordingly. The results in Chapter 4 show that duck ovodefensins are expressed most highly in the shell gland. It could therefore be theorised that the wet environments encountered by

waterfowl result in greater antimicrobial requirement for the egg shell since bacterial contact is likely heightened, however this does not explain the high expression of turkey ovodefensins in the shell gland. Of course environmental observations alone cannot prove the hypothesis and indeed even with a large number of ovodefensins it would be difficult to do so as environmental pressures are often multifaceted and complex. Nonetheless the ongoing ‘arms race’ between host and pathogen is a strong driver of evolution and immune genes such as AMPs are often under positive selection and therefore evolve more rapidly (Kosiol et al., 2008). Therefore particular pathogens of the host environment may be playing a role in how the ovodefensins within any given species evolve. In this thesis it was only possible to test the antimicrobial capabilities of a few peptides against a small range of bacterial species but it was evident that the spectrum of activity and efficacy varied. It would be interesting to test a much wider range of peptides and microbial species in order to determine if any connection could be made between species and activity. It would be of particular interest to test ovodefensins against known pathogens isolated from the host species. However this could prove problematic, particularly when trying to obtain strains from species such as the zebra finch. By increasing the range of peptides and microbes tested it may also be possible to shed light on how factors such as charge and spacing affect activity. In this respect the large number and diversity of naturally occurring ovodefensins presents an interesting opportunity to study functional evolution. On the other hand the fact that both sequence and motif spacing appear to be important features of these molecules also provides an interesting and novel approach in designing variants with improved activity.

In vitro ovodefensin activity appears to be fairly limited, however *Gallus gallus* OvoDA1 was the most potent peptide in this investigation demonstrating a large reduction in both *E. coli* and *S. aureus* and showing little sensitivity to factors such as salt concentration and pH. It would therefore seem appropriate to use *Gallus gallus* OvoDA1 as a template for designing variants with improved activity. In addition since this work was carried out the structure of *Gallus gallus* OvoDA1 has

been resolved using 3D-NMR (Herve et al., 2014) which provides the knowledge to make more informed changes to the molecule.

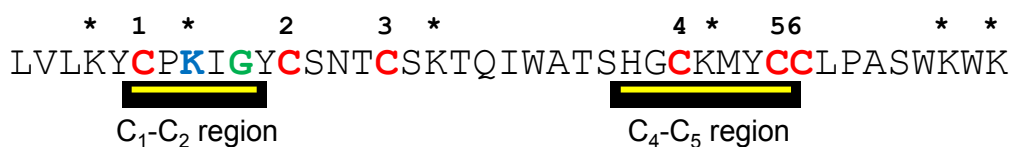


Figure 7.1: Primary sequence of *Gallus gallus* OvoDA1. Conserved cysteine residues are shown in red and numbered, exposed lysine residue K8 in blue and conserved glycine residue G10 in green. Regions between C1-C2 and C4-C5 are underlined. The charged lysines are indicated by '*'.

All defensin molecules contain six conserved cysteine residues which form three disulphide bridges (Ganz, 2003). NMR analysis indicates that in *Gallus gallus* OvoDA1 these bridges are formed in a C1-C5, C2-C4 and C3-C6 arrangement (Herve et al., 2014) which is the same structure that is observed in all other classical vertebrate defensin molecules (Selsted and Ouellette, 2005). A recent paper by Schroeder et al (2011) revealed that the reduction of human β -defensin 1 bonds unmasked potent antibacterial action and it would therefore seem sensible to determine the contribution of such bridges to *Gallus gallus* OvoDA1 activity. In order to better understand how disulphide bonds affect activity it would be interesting to remove each of the cysteine pairs, this could be done by substituting cysteine with alanine which has similar properties and testing the peptide's activity *in vitro*. Moreover whilst discovering novel ovodefensin homologs it was possible to show that a previously studied turtle peptide belonged to this family. This peptide belongs to the OvoDB subfamily and the previous study suggested that it has cysteine bonding that may not be classical (C1-C6, C2-C5, C3-C4) (Chattopadhyay et al., 2006). It is unknown how this affects activity or if other OvoDB members also possess this arrangement. It would therefore be interesting to determine the structure

and arrangement of disulphide bonds of other OvoDB members such as *Gallus gallus* OvoDB1 and ultimately the disulphide arrangement of all the sub-families.

The differences in spacing are ultimately due to differing numbers of amino acids between the cysteine residues. The C1-C2 region varies among ovodefensin molecules and the wider family of classical avian β -defensins. Within this region there is a highly conserved glycine residue, in *Gallus gallus* OvoDA1 the glycine is one residue from C1 whereas it is 3 residues from C1 in *Gallus gallus* OvoDB1 which shows diminished activity. It would therefore be interesting to synthesise peptides based on *Gallus gallus* OvoDA1 in which the spacer between C1 and the conserved glycine is reduced in length sequentially from three to zero and assess how this affects activity and conformation. The C1-C2 region of *Gallus gallus* OvoDA1 also contains a highly charged and hydrophilic lysine residue (K8) that protrudes from a region of otherwise intermediate hydrophobicity (Herve et al., 2014). As charge is a varying factor in ovodefensins it would be interesting to study the effect of replacing the lysine residue with an uncharged hydrophobic alanine residue.

The C4-C5 region in *Gallus gallus* OvoDA1 is preceded by two residues (HG) which are absent in classical β -defensins. This addition results in two short parallel β -sheets in place of the classical alpha helical N-terminus traditionally seen in β -defensins (Herve et al., 2014). It would therefore be of interest to synthesise a *Gallus gallus* OvoDA1 molecule lacking these residues and determine if this affects conformation, disulphide bonding and activity. The spacing within the C4-C5 region also varies from 3 to 4 residues among the ovodefensin sub-families and 5-6 residues in classical β -defensins. Changing the spacing of this region in *Gallus gallus* OvoDA1 from 3 to 6 residues by the sequential addition of alanine would provide insight into the role of the structural features which distinguish the ovodefensin family from classical β -defensins.

Finally charge varies from +4 to +10 within the currently identified ovodefensin peptides yet there was no clear relationship between charge and activity in the antibacterial studies outlined in chapter 5. As ovodefensin activity *in vitro* is thought to rely on electrostatic interactions with the bacterial membrane (Gong et al., 2010) it

would be interesting to see the effect of altering the charge of *Gallus gallus* OvoDA1 without changing its structure. This could be achieved by replacing the lysines at positions 18 and 39 (Figure 1) with alanine molecules resulting in a change of charge from +6 to +3.

Ultimately a greater understanding of the relationship between structure and function could lead to the production of a pipeline of antimicrobial peptides with improved activity. These could form the basis of a new group of “antibiotics” or to be more precise “antimicrobials” which is so desperately needed in this current era of antibiotic resistance both in the agricultural industry and also the human health and veterinary sectors.

7.1.1.3. Peptide feed additives

The data presented in Chapter 6 showed an impressive improvement in mean body weight gain when broilers were fed ovodefensin peptide in the feed. This result was supported by an improvement in FCR. Ovodefensin fed broilers also had an altered gut microflora; in particular there was a highly significant reduction in *Clostridium perfringens* in the ileum. *C. perfringens* is the causative agent of necrotic enteritis and of major importance to the poultry industry (McDevitt et al., 2006, Dahiya et al., 2006). Particularly since the withdrawal of antibiotics in 1999 a reduction of this organism is likely to be well received. Also of note was a reduction in the opportunistic pathogen *E. coli* in the caeca and a reduction in caecal *Bifidobacteria*. Although *Bifidobacteria* is generally considered part of a healthy gut microbiota it primarily metabolises simple sugars and is therefore more prevalent earlier in the digestive tract (Rinttila and Apajalahti, 2013). Large numbers of *Bifidobacteria* and *Lactobacillus* in the caeca are indicative of poor nutrient absorption in the small intestine therefore a reduction is indicative of improved absorption which may have contributed to the increased body weight. Another important observation from the peptide feed trial was an increase in caecal Clostridial clusters, particularly cluster IV. These Clostridial clusters are considered to be beneficial for animals (Rinttila and Apajalahti, 2013) as they produce butyric acid as an end product of metabolism

(Pryde et al., 2002). Butyric acid is generally considered to be synonymous with good gut health and has been implicated in improved motility and gut barrier integrity (Pryde et al., 2002). There is also some suggestion that butyrate stimulates the expression of endogenous defensin activity (Sunkara et al., 2011); the SCFA analysis supported an increase in butyric acid in the caeca. The SCFA analysis also revealed an increase in acetic acid in the caeca; this may be an indication of an increase in heterofermentative *Lactobacillus* in the intestine. Homofermentative fermentation results in >85% lactic acid production whereas heterofermentative metabolism results in the production of both lactic acid and acetic acid in equimolar concentrations (Ganzle and Follador, 2012). Although both types of *Lactobacillus* are indicative of good gut health there is some evidence that heterofermentative metabolism is correlated with improved performance (Lidbeck et al., 1992).

The effect of ovodefensins in the *ex vivo* gut model and in the *in vivo* feed trial was substantial, particularly when looking at growth and shifts in the microbiome. However the *in vitro* experiments, which measure the ability of the peptide to kill bacteria directly through membrane disruption was not predictive of such large effects. It therefore seems unlikely that this mode of action was solely responsible for the impressive effects observed in these experiments and in fact there is some debate as to whether host defence peptides are directly antimicrobial *in vivo* at lower concentrations (Bowdish et al., 2006). However, it is now widely accepted that defensins have a wide range of immune-modulatory activities and have been implicated in processes such as re-epithelisation and proliferation, chemokine production and chemotaxis and interactions with effector cells of the innate immune response (Bowdish et al., 2006). There are some animal infection models which demonstrate a reduction in bacterial counts when host defence peptide expression is either introduced or increased. For example when mice were challenged with *Pseudomonas aeruginosa* infection after lung transfer of LL-37/hCAP-18 there was an observed reduction in bacterial counts and inflammatory cytokine, TNF- α production (Bals et al., 1999). However an improved phenotype is not always associated with reduced bacterial counts. For example in another *P. aeruginosa*

mouse lung study the instillation of either hBD2 or a LL-37 derivative did not lead to reduced bacterial counts despite a reduction in lung damage and pro-inflammatory cytokine production (Sawa et al., 1998). The *in vivo* experiments from this trial show a change in the microflora, but unfortunately there was insufficient time to study changes in the host immune response which may have been responsible for the bacterial shift. Although, as bacteria can also directly affect the immune system it may be difficult to determine what was cause and effect. However, whatever the case, it is apparent that it is advantageous for pathogenic bacteria to subvert the expression of host defence peptides and decreased expression is often correlated with disease occurrence and severity (Bowdish et al., 2006). For example both LL-37 and hBD2 expression are depressed in patients with atopic dermatitis (Ong et al., 2002) and LL-37 has been shown to be decreased in *Shigella* infection (Islam et al., 2001). Therefore if the increase in butyrate observed in this study does lead to an increase in endogenous defensin expression as predicted (Sunkara et al., 2011) then this may be advantageous to the host in controlling bacterial pathogens.

Similarities between host defence peptides and chemokines have been documented, indeed antimicrobial activity has been observed in many chemokines and certain HDPs have chemotactic activity (Bowdish et al., 2006). In fact it has previously been proposed that certain HDPs may have evolved from the duplication of chemokines, however this connection is considered controversial (Yang et al., 2002, Duerr and Peschel, 2002). Chemotactic activity in HDPs has been shown to be diverse and varies between peptides. For example both HNP1 and HNP2 can induce chemotaxis of monocytes; however HNP1 is a more potent chemoattractant than HNP2 and conversely HNP3 does not demonstrate any significant chemotaxis at all (Territo et al., 1989). Both β -defensin hBD1 and hBD2 are chemoattractants for memory T-cells and immature dendritic cells (Yang et al., 1999), and LL-37 has been demonstrated to be chemotactic for many cell types across multiple species including rat mast cells (Niyonsaba et al., 2002) and human neutrophils and monocytes (De et al., 2000). This diversity demonstrates the complexity of HDP chemotaxis and therefore different peptides are likely to be mediated through a

number of receptors and pathways. Indeed if chemotaxis plays a role in ovodefensin activity in the peptide trials this will make understanding the mechanisms behind this difficult. However, samples of the intestine which have been fixed for histology may reveal the increased presence of particular cell types such as heterophils and macrophages and give a starting point for investigation.

There is a large body of literature demonstrating the interaction of HDPs with effector cells of the innate immune response such as natural killer and mast cells. Monocytes and macrophages when stimulated with LPS or pro-inflammatory mediators induce the production of HDPs by epithelial cells and keratinocytes (Liu et al., 2003); but in turn monocytes and macrophages are also responsive to HDPs (Bowdish et al., 2006). Host defence cells have also been observed to interact with epithelial cells, for example in the induction of proliferation (Nishimura et al., 2004). The target of in-feed ovodefensin peptides is the small intestine where there is an abundance of epithelial cells and effector molecules. To date there has been no work conducted as to the immune-modulatory abilities of ovodefensins and further investigation of gene expression and enumeration of innate immune cells may give an indication of mechanisms at work in the intestine. Indeed, although the work carried out in this thesis did not look at ovodefensin activity in the natural setting, understanding the immune-modulatory capabilities in these trials may be informative of their natural role in the oviduct and the egg.

Overall the results from the feed trial are extremely positive and suggest that ovodefensin feed additives may provide an interesting alternative to antibiotic growth promoters. However in order for peptide feed additives to be considered a viable AGP replacement they would need to be accepted by regulatory bodies and the poultry industry. Current EU directives impose a complete ban on the use of antibiotics as a prophylactic feed additive, therefore ovodefensin feed additives would have to be accepted as a separate class. The European Commission (EC) regulation No 429/2008 states that ‘antibiotic’ means antimicrobials produced by, or derived from, a micro-organism, which destroys or inhibits the growth of other micro-organisms. Whereas the alternative characterization ‘antimicrobials’ means

substances produced either synthetically or naturally, used to kill or inhibit the growth of micro-organisms, including bacteria, viruses or fungi, or of parasites, in particular protozoa. As these peptides are natural peptides produced in fungi they clearly fall into the antimicrobials category and would therefore likely be accepted by the regulatory bodies. The fact that these peptides are naturally occurring in eggs, routinely consumed by humans and not genetically modified in any way means that they are also likely to be widely accepted by both the poultry industry and consumers alike.

Despite the fact that in-feed addition of ovodefensin peptide produced significant benefits to poultry performance it is important that their use is properly controlled as part of a good management strategy. No sole intervention can successfully control disease whilst enabling the high performance and fast growth of broilers as well as maintaining high welfare standards. Any AGP-like treatment should be considered as part of a wider programme ensuring good husbandry, diet, housing and welfare.

7.1.2. Directions for future research

Initial experiments outlined in this thesis demonstrate the potential of antimicrobial peptides to modulate the gut microbiome and improve broiler performance when administered in the feed. This discovery has enormous potential for commercialisation, but before this could be achieved further work would need to be carried out to determine whether these observations are reliably reproducible. In addition to this it would be desirable to work out the best dosing and duration strategies for optimal performance at the lowest possible cost to ensure the product is economically viable in an industry where profit margins are extremely tight. In the final part of this chapter areas of research will be discussed that may be important for progressing antimicrobial peptide feed additives both in terms of creating a viable pipeline of products and also in understanding the mechanisms behind their activity.

Firstly, a full biological replicate of the data presented in Chapter 6 should be conducted to confirm the growth promoting activities of the ovodefensin family. In this repeat experiment it would be interesting to include the supernatant control as in

the *ex vivo* gut model to determine if the beneficial effects observed are due to the peptide or background fungal components which are not removed during the purification stage. In the initial experiment the birds were culled at 21 days and as broilers are commercially reared to between 35 and 42 days the beneficial effects should be observed for the duration of this period. However, for an antimicrobial peptide feed additive to be commercially viable it is essential that it is cost effective therefore feeding the peptide for the duration of the rearing period may prove too expensive. The first few days of life are considered critical for establishing a beneficial microbiota (Rinttila and Apajalahti, 2013) and therefore it may be possible to feed the peptide in the early stages of growth and still see benefits long after withdrawal. Broilers are normally fed a starter diet for approximately the first 11 days before the feed is changed to a grower diet; only including peptide in the starter diet would allow transient feeding without any additional labour. Human diet has been shown to affect the gut microbiota but when the diet is altered the response in microbial composition to this change can be delayed (Maslowski and Mackay, 2011). If the same is true in chickens the fact that they are only reared for an additional 4 weeks after the proposed withdrawal period would hopefully mean that the microbiota would not have time to revert back to 'untreated'. Indeed it may be that the beneficial microbiota developed in this early stage is able to out-compete opportunistic pathogens, which is the premise behind currently used competitive exclusion products (Lee et al., 2010).

In the experiments carried out in this thesis an interesting observation was made with *Taeniopygia guttata* OvoDB1. It appeared that the lowest dose of this peptide produced effects as beneficial as the highest dose and in fact much greater than the middle dose. This observation may purely be down to chance or it may be that the middle dose did not perform as expected. However if the biological replicate were to reproduce this effect then the possibility of feeding peptides at lower doses should be investigated. In fact it is recommended that in the biological replicate a lower dose of *Taeniopygia guttata* OvoDB1 is included. Being able to reduce the dose of a peptide has clear financial gain.

In section 7.1.1.2 the creation of variants of known ovodefensin peptides was proposed in order to produce an ‘optimal ovodefensin’. If any of these variants showed greater performance in the *in vitro* tests or *ex vivo* gut model it would be interesting to determine whether improved performance in these tests resulted in improved performance *in vivo*. It would also be of interest to see whether combinations of peptides such as *Gallus gallus* OvoDA1 and TENP which are proposed to function through very different mechanisms would produce a synergistic effect when fed together perhaps reducing the overall quantity of peptide required and thus in turn reducing the cost.

Finally as the overall purpose of this experiment was to produce an alternative to traditionally used antibiotic growth promoters then ideally these peptide products should be compared to AGPs in the same experiment to compare their efficacy. When the ideal dose and feeding strategy has been identified it would be interesting to compare its performance against a known AGP such as bacitracin.

Within this thesis it was demonstrated that the in-feed inclusion of ovodefensin peptides could modulate the gut microbiota and improve FCR and growth performance. However the mechanisms behind this observation remain to be elucidated. It is widely documented and accepted that as well as the ability to directly kill bacteria through the formation of pores in the cell membrane, defensins are able to modulate the immune system and as such this would make an interesting area to study.

One approach to understanding the underlying mechanisms of improved growth would be to analyse intestinal gene expression in the birds with the greatest performance and compare it with the controls. For a limited subset of birds RNA-Seq analyses of ileal and caecal tissues should be conducted to observe global changes in the transcriptome. By analysing expression globally it may be possible to detect changes not only in the immune response but also in other parameters that could affect the growth of the birds such as expression of molecules involved in digestion and nutrient uptake e.g. insulin and glucagon. RNA-Seq is an expensive

tool and as such it would only be reasonable to look at a small subset of birds, however as currently very little is known about how growth promotion on the gut works it would not be feasible to take a more targeted approach. However the information gathered could then be used to target particular genes or specific pathways of interest in a larger subset of birds using less expensive methods such as quantitative PCR. One family of genes that would be of particular interest are the classical β -defensin genes expressed in the intestine. This study observed an increase in the butyrate producing clostridial clusters and ultimately an increase in butyrate concentration in the animals treated with Ovodefensin peptides. Butyrate is often associated with good gut function and health, but more recently it has been suggested that butyrate increases the endogenous expression of some defensin genes (Sunkara et al., 2011). If this were the case in this study it would be a clear example of an indirect mechanism of immune modulation by antimicrobial peptide feed additives. Additionally, analysing gene expression in other regions of the gastrointestinal tract, particularly earlier steps in the digestive pathway, may prove insightful as the peptides may be affecting a very early stage of digestion which could be missed in the ileal and caecal samples.

In addition to evaluation of gene expression it would be interesting to determine if in-feed inclusion of ovodefensin peptides impact on gut morphology. It has been documented that traditional antibiotic growth promoters alter villi density, area and crypt depth and can also alter the thickness of the *mucularis mucosa* in poultry (Miles et al., 2006). Other factors such as epithelial cell density may also change which in turn may affect gut function and the resistance of the bird to pathogens. Fixed samples from the ileum and caeca of birds with a significant increase in weight gain could be stained with haematoxylin and eosin for comparison of gross histology and immune cell numbers with the control birds. The authors of a recent study fed small cationic peptides to birds and observed an effect on heterophil and monocyte function (Kogut et al., 2012). It would be interesting to see if ovodefensin peptides were also able to stimulate phagocytosis and oxidative burst in the host cells as these functions contribute to the innate defence of the bird. In addition to this the birds in

the field experiment described in Chapter 6 were vaccinated with a commercial Mareks vaccine at one day of age. It would be possible, using serology techniques, to measure vaccine titres in the birds as another potential indicator of differences in immune function in the ovodefensin treated birds.

Whatever the mechanisms behind the growth promoting effects of ovodefensin peptides they clearly offer an exciting avenue of exploration in finding an alternative to antibiotic growth promoters in the poultry industry. Research into optimising this effect and understanding the mechanisms behind it are therefore of great interest to the poultry industry and also for those wishing to understand the biology behind HDP activity.

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Appendix

Appendix 1

Reagents and solutions

Phosphate buffered saline (pH adjusted)

200 mM Na_2HPO_4 , 200 mM NaH_2PO_4 , 100 mM NaCl

pH was adjusted by the addition of NaH_2PO_4 to Na_2HPO_4 until pH 6.4, 7.4 or 8.4 was obtained.

Phosphate buffered saline (Salt adjusted)

200 mM Na_2HPO_4 , 200 mM NaH_2PO_4 , * NaCl ; pH 7.4

* NaCl was added at a final concentration of 50, 100 or 150 mM.

Ileal buffer

0.02 M K_2HPO_4 , 0.02 M $\text{NH}_4\text{H}_2\text{PO}_4$, 0.0006 M MgSO_4 . pH 6.5.

Caecal buffer

0.02 M K_2HPO_4 , 0.02 M $\text{NH}_4\text{H}_2\text{PO}_4$, 0.0006 M MgSO_4 . pH 6.5.

To 1 litre of caeca buffer add 0.3125 g L-cysteine and 0.3125 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$

Appendix 2

Peptide information provided to Roal to aid in recombinant peptide production.

Gallus gallus OvoDA1 (Gallin)

Source

Chicken

cDNA sequence

CTGGTCCTGAAGTACTGCCCCAAGATCGGCTACTGCTCCAACACGTGCTC
CAAGACACAGATCTGGGGCCACCTCCCACGGATGCAAGATGTACTGCTGC
CTGCCTGCCAGCTGGAAGTGGAAATAA

Amino acid sequence

LVLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLPASWKWK.

PEPSTATS using Emboss

Molecular weight = 4731.68 Residues = 41

Average Residue Weight = 115.407 Charge = 6.5

Isoelectric Point = 9.1165

Probability of expression in inclusion bodies = 0.858

Residue	Number	Mole%	DayhoffStat
A = Ala	2	4.878	0.567
B = Asx	0	0.000	0.000
C = Cys	6	14.634	5.046
D = Asp	0	0.000	0.000
E = Glu	0	0.000	0.000
F = Phe	0	0.000	0.000
G = Gly	2	4.878	0.581
H = His	1	2.439	1.220
I = Ile	2	4.878	1.084
J = ---	0	0.000	0.000
K = Lys	6	14.634	2.217

L = Leu	3	7.317	0.989
M = Met	1	2.439	1.435
N = Asn	1	2.439	0.567
O = ---	0	0.000	0.000
P = Pro	2	4.878	0.938
Q = Gln	1	2.439	0.625
R = Arg	0	0.000	0.000
S = Ser	4	9.756	1.394
T = Thr	3	7.317	1.200
U = ---	0	0.000	0.000
V = Val	1	2.439	0.370
W = Trp	3	7.317	5.629
X = Xaa	0	0.000	0.000
Y = Tyr	3	7.317	2.152
Z = Glx	0	0.000	0.000

Property	Residues	Number	Mole%
Tiny	(A+C+G+S+T)	17	41.463
Small	(A+B+C+D+G+N+P+S+T+V)	21	51.220
Aliphatic	(A+I+L+V)	8	19.512
Aromatic	(F+H+W+Y)	7	17.073
Non-polar	(A+C+F+G+I+L+M+P+V+W+Y)	25	60.976
Polar	(D+E+H+K+N+Q+R+S+T+Z)	16	39.024
Charged	(B+D+E+H+K+R+Z)	7	17.073
Basic	(H+K+R)	7	17.073
Acidic	(B+D+E+Z)	0	0.000

Other

Antimicrobial activity demonstrated against *E.coli*; 50% inhibition at 0.25 μ M and 95% inhibition at 1 μ M

Cysteine bonds = 1-5, 2-4, 3-6.

Proposed mode of action = pore formation due to electrostatic interaction.

Taeniopygia guttata OvoDB1 (Taeniopygin 2)**Source**

Zebra finch

cDNA sequence

CAGCCAAAAAGGAGCTGCAGAGGGCATTGCTCCAGGACCTGTGGCAAAG
 GAGAGAGGGAGGAGCACACTGAGGACTGCGGAGGGATGCACTGCTGCCT
 GACACACAGGAAAAGGAAGTAG

Amino acid sequence

QPKRSCRGHCSRTCCKGEREEHTEDCGGMHCCLTHRKRK.

PEPSTATS using Emboss

Molecular weight = 4499.12 Residues = 39

Average Residue Weight = 115.362 Charge = 7.0

Isoelectric Point = 8.9019

Probability of expression in inclusion bodies = 0.811

Residue	Number	Mole%	DayhoffStat
A = Ala	0	0.000	0.000
B = Asx	0	0.000	0.000
C = Cys	6	15.385	5.305
D = Asp	1	2.564	0.466
E = Glu	4	10.256	1.709
F = Phe	0	0.000	0.000
G = Gly	5	12.821	1.526
H = His	4	10.256	5.128
I = Ile	0	0.000	0.000
J = ---	0	0.000	0.000
K = Lys	4	10.256	1.554
L = Leu	1	2.564	0.347
M = Met	1	2.564	1.508
N = Asn	0	0.000	0.000

O = ---	0	0.000	0.000
P = Pro	1	2.564	0.493
Q = Gln	1	2.564	0.657
R = Arg	6	15.385	3.140
S = Ser	2	5.128	0.733
T = Thr	3	7.692	1.261
U = ---	0	0.000	0.000
V = Val	0	0.000	0.000
W = Trp	0	0.000	0.000
X = Xaa	0	0.000	0.000
Y = Tyr	0	0.000	0.000
Z = Glx	0	0.000	0.000

Property	Residues	Number	Mole%
Tiny	(A+C+G+S+T)	16	41.026
Small	(A+B+C+D+G+N+P+S+T+V)	18	46.154
Aliphatic	(A+I+L+V)	1	2.564
Aromatic	(F+H+W+Y)	4	10.256
Non-polar	(A+C+F+G+I+L+M+P+V+W+Y)	14	35.897
Polar	(D+E+H+K+N+Q+R+S+T+Z)	25	64.103
Charged	(B+D+E+H+K+R+Z)	19	48.718
Basic	(H+K+R)	14	35.897
Acidic	(B+D+E+Z)	5	12.821



Comparative biology and expression of TENP, an egg protein related to the bacterial permeability-increasing family of proteins

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ABSTRACT

The 'transiently expressed in neural precursors' (*TENP*) gene product is a member of the bacterial/permeability-increasing (BPI) family of antimicrobial proteins but was first identified as having a role in an early neurological event occurring in post-mitotic cells. However, recent characterisation of the egg white proteome has shown that *TENP* is an important egg component constituting ~0.1–0.5% of the total protein and suggesting it is expressed in the adult oviduct. In this study we confirmed quantitatively that the expression of *TENP* is largely confined to the tubular glands of the magnum of the oviduct, where egg white synthesis occurs, with around 10,000 times more expression than in the embryo where *TENP* was first identified. *TENP* expression is significantly increased with the administration of oestrogen or progesterone ($P < 0.001$) and is reduced in regressed oviducts ($P < 0.001$) demonstrating gonadal steroid control, typical of an oviduct and egg specific gene. A putative translational start site for *TENP* has been characterised and the evidence indicates that it is expressed as one predominant transcript. In comparison with the published sequence, insertion and deletion events have been identified causing a partial frame-shift that results in an altered amino acid sequence to that previously documented. *TENP* is conserved across divergent avian species being found in chicken, turkey, duck and zebra finch and its expression profile confirmed in both chicken and duck. Similarity searches have shown homology with the BPI-like family of innate immune genes, particularly with palate, lung and nasal epithelial clone (*PLUNC*) members of this family. We therefore believe that at least in adults the role of *TENP* is as a major component of egg, particularly the white and it is probable that it contributes to its antimicrobial function.

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1. Introduction

The egg must provide an embryo with the nutrients it needs to grow as well as protection from pathogens to ensure it survives to hatch. One way in which this is achieved is utilising antimicrobial proteins and peptides, molecules of the innate immune system (Wellman-Labadie et al., 2007). The current requirement to find new strategies to combat infection makes the study of new antimicrobial proteins extremely valuable and also aids our understanding of how the avian egg manages to prevent contamination during incubation (Kovacs-Nolan et al., 2005).

A large amount of work has been made on proteins in the egg, indeed investigators have utilised the prodigious expression of egg

protein genes, especially ovalbumin, to advance the understanding of gene expression (McKnight, 1978). However, that very feature has masked the identity of many of the components of the egg. Recently proteomic studies have started to uncover some of the secrets that the large quantities of ovalbumin were hiding (Mann et al., 2008). The *TENP* gene appears to be an example of one such protein. The *TENP* (transiently expressed in neural precursors) gene was first identified in the brain and retina of developing neural tissues of chickens using reverse transcription polymerase chain reaction (RT-PCR), and was proposed to function in an early neurological event occurring in post-mitotic cells before they enter the stage of overt differentiation (Yan and Wang, 1998). The 47 kDa protein has since been identified during proteomic studies of chicken egg white, vitelline membrane, shell and yolk (D'Ambrosio et al., 2008; Farinazzo et al., 2009; Guerin-Dubiard et al., 2006; Mann, 2007, 2008; Mann and Mann, 2008; Mann et al., 2006). *TENP* has also been identified in proteomic analysis of emu egg white (Maehashi et al., 2010) showing its conservation across divergent avian species.

Similarity searches have shown homology of *TENP* with the bactericidal/permeability-increasing (BPI)-like family of innate immune genes (Mulero et al., 2002), which are found in adjacent chromosomal regions to *TENP* on chromosome 20 (Chiang et al., 2011). Computer

Abbreviations: *TENP*, transiently expressed in neural precursors; BPI, bacterial/permeability-increasing; PLUNC, palate, lung and nasal epithelial clone; RT-PCR, reverse transcription polymerase chain reaction; LPS, lipopolysaccharide; BNF, buffered-neutral formalin; DES, diethylstilbestrol; TBST, tris-buffered saline, tween 20; BSA, bovine serum albumin; HRP, horse radish peroxidase; OPD, O-Phenylenediamine; IHC, immunohistochemistry.

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analysis of the primary sequence of *TENP* predicted three putative transmembrane helices suggesting it may be a membrane protein (Yan and Wang, 1998). This was supported by the immunodetection of *TENP* in cellular membrane fractions after expression in chicken embryonic fibroblast cells (Yan and Wang, 1998). However, it has been suggested that *TENP*, as previously proposed for BPI (Beamer et al., 1998), may be membrane associated rather than an integral membrane protein. It has been suggested that *TENP* is a divergent ortholog of human *LPLUNC2* (Chiang et al., 2011). The PLUNC (palate, lung and nasal epithelial clone) protein family are structural homologues of BPI proteins (Chiang et al., 2011) and are divided into two groups; short (SPLUNC) and long (LPLUNC) proteins. SPLUNCs contain a region structurally homologous to the BPI N-terminal domain whereas LPLUNCs contain domains similar to both the BPI domains (Bingle and Craven, 2002). The N-terminal domain of BPI is responsible for lipopolysaccharide (LPS) and bacterial binding as well as endotoxin neutralisation and antibacterial cytotoxicity whereas the C-terminal domain is associated with opsonic effects thus enhancing phagocytosis (Schultz et al., 2001). The *TENP* molecule detected in the embryo has two distinct regions that are analogous to the BPI1 (N-terminal) and BPI2 (C-terminal) domains of the BPI protein. There is currently little evidence from EST data (Chicken (*Gallus gallus*) Genome Browser Gateway, 2013) to support a full length *TENP* transcript in adult birds which would allow both the BPI-like domains present to be expressed in one molecule; however the proteomic evidence supports both domains being present in egg white (Karlheinz Mann, personal communication, November 11, 2010). It has been proposed that the function of *TENP* is associated with the innate defence of eggs against pathogens (D'Ambrosio et al., 2008; Guerin-Dubiard et al., 2006; Mann, 2007) however its proposed function is yet to be experimentally proven and is largely based on its relationship to the BPI-like family. A chicken member of the BPI/PLUNC superfamily is found in the egg shell and has previously been shown to possess modest antibacterial activity against a range of gram positive and gram negative bacteria (Gautron et al., 2011). It is also documented that this protein is able to bind *Escherichia coli* LPS in vitro (Gautron et al., 2011) and its expression in the infundibulum is up-regulated after systemic administration of this molecule (Bedrani et al., 2013) strongly supporting a role in egg antimicrobial defence. Although mammalian members of this family have been documented to interact with LPS this has been demonstrated to be both pro and anti-inflammatory and the family's role in host defence against bacteria is still to be fully elucidated (Bingle and Craven, 2004). However, due to the large number of known antimicrobial proteins, including Ovocalyxin 36, already identified in the egg an antimicrobial role would seem plausible.

The overall aim of this study was to determine the expression of *TENP* in different tissues and physiological states to support the hypothesis that its role is as a specific egg protein in addition to its proposed developmental role. This study has defined the transcriptional start site and putative translational start site and also investigated the evolutionary relationship of *TENP* in a number of bird species and demonstrated the pattern of expression in two divergent avian species, the chicken and duck.

2. Methods

2.1. Bioinformatic analysis

The *TENP* protein sequence (NCBI accession no. AF029841) was used to perform a BLAT search (Kent, 2002) of the May 2006 release of the chicken EST database using the default parameters of the UCSC genome browser (Chicken (*Gallus gallus*) Genome Browser Gateway, 2013) to identify ESTs and sequences related to the locus. The Staden package (Staden, 1996) was used to build a consensus sequence using the ESTs available. This allowed a gap present in the genome build to be bridged and allowed a comparison to be made between the genomic, EST and published *TENP* mRNA sequences in order to address differences in sequence identity. Primer3 (2013) was used to design primers (Table 1) for re-sequencing across the length of the consensus sequence including a putative alternative transcriptional start site identified in the consensus sequence. Signal P (Bendtsen et al., 2004) was used to assess the likelihood of a signal peptide produced by each translational start site. Exon contributions were estimated from genomic DNA using BDGP NNSPLICE version 0.9 (Reese et al., 1997) (Table 2).

The duck genome database (*Anas platyrhynchos* – version 1) (Ensembl Pre-release Genome Browser, 2013) and turkey genome database (*Meleagris gallopavo* – assembly UMD2) (Ensembl Genome Browser, 2013) were searched using TBLASTN for potential homologues using the 629 amino acid mature *TENP* protein. Primers were designed using Primer3 (2013) to amplify both the potential turkey and duck *TENP* sequences from cDNA (Table 1).

2.2. Animals and tissue collection

2.2.1. Tissue panel

To determine *TENP* expression the following tissues were taken from sexually mature White Leghorn LSL hens (*Gallus gallus*) (Lohmann): Oviduct (magnum, shell gland, isthmus, vagina), ovarian stroma, crop, duodenal loop, gizzard, caeca, cloaca, lung, adrenals, cerebellum, retina, spleen, liver, kidney, and heart (n = 4). After dissection tissue was placed in RNA later (Ambion, Applied Biosystems, Warrington, UK) and stored at 4 °C overnight before storage at –80 °C. Samples weighed no more than 100 mg.

Sexually mature Pekin ducks (*Anas platyrhynchos*) (Cherry Valley): Oviduct (magnum, shell gland, isthmus, vagina), ovarian stroma, crop, proventriculus, small intestine, duodenal loop, gizzard, large intestine, caeca, cloaca, gall bladder, lung, trachea, pituitary, adrenals, cerebellum, hypothalamus, tongue, spleen, breast muscle, liver, kidney and heart. Samples were stored as above.

2.2.2. Embryonic tissue

Brain, retina and heart tissue were taken from embryonic day 10 (E10) (n = 6) and day 16 White Leghorn chickens (E16) (n = 6). E10 and E16 correspond with the Hamburger–Hamilton (HH) embryonic

Table 1
Names and sequences of primers used in the study.

Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence
TENP Exon1F1	AGGATGGGAACAGCAACAG	TENP End1	ATCCTCCTTCTGCACCAAAA
TENP RTF	CACGTGCTGGAGGAGCTGTT	TENP RTR	ACAACGTTGACGTCGGTGTA
D.TENP F3	AGACCATACGCAGAGGTGGT	D.TENP R3	AGGTTGCACAGGAGCAAGAC
D.TENP F8	AGGGAATCACCATGTCCTTG	D.TENP R10	TGATCTTCTGTGCCATGGTG
T.TENP F1	GTCTCAAAGCCACATGCAGA	T.TENP R1	GGTTTCCACTGCTTTCACC
		SP1	ATCTGGAGGACTTGCTTTC
		SP2	CCCATGCTACGTGGAGGTC

Table 2

Exon contributions. Exons are shown as nucleotide contributions in relation to the *TENP* sequence produced in this study (EMBL HG007958).

Exon No.	Exon Contribution
1	1–53
2	54–170
3	171–270
4	271–375
5	376–510
6	511–556
7	557–617
8	618–709
9	710–885
10	886–928
11	929–1093
12	1094–1161
13	1162–1207
14	1208–1271
15	1272–1348
16	1349–1576

developmental stages HH36 and HH42 respectively. Samples were stored as above.

2.2.3. Time of oviposition

Magnum tissue was obtained from sexually mature White Leghorn hens with an ovum at various positions in the oviduct, see [Gong et al. \(2010\)](#) for details. Briefly, magnum tissue was processed either when the egg was in the magnum ($n = 5$), in the shell gland where the stage of calcification was determined by electron microscopy and recorded as early ($n = 8$), mid ($n = 9$) and late ($n = 10$) calcification or during a pause day ($n = 11$) when there was no evidence of ovulation. Tissues for immunolocalisation studies (magnum, isthmus, shell gland and caecum) were harvested from 5 of these laying hens post mortem and fixed in 10% buffered-neutral formalin (BNF) for 24 h prior to being processed to paraffin wax using a 16 hour processing cycle in a Thermo Shandon Excelsior tissue processor.

2.2.4. Effect of oviduct development

In domestic chickens, the onset of incubation behaviour is characterised by the regression of the oviduct due to the withdrawal of gonadotrophic support ([Dunn et al., 1996](#)). This natural phenomenon was exploited to determine if *TENP* expression differed between in lay hens ($n = 11$) and those where the oviduct had regressed ($n = 11$). Magnum and shell gland tissue was collected from hens of a Silkie \times White Leghorn cross, after dissection tissue was frozen in liquid N_2 and stored at -80°C . Samples weighed no more than 100 mg.

2.2.5. Northern analysis

To determine definitively the expression of *TENP* isoforms a northern analysis was carried out using magnum from in-lay layer ($n = 3$) and broiler ($n = 3$) lines. Broiler liver was included as a negative control. After dissection, tissue was placed in RNA later (Ambion, Applied Biosystems, Warrington, UK) at 4°C overnight then stored at -80°C .

2.2.6. Induction of *TENP* with steroid hormones

The induction of *TENP* with steroid hormones was adapted from a method described previously by [Kunnas et al. \(1992\)](#). Three week old ISA brown chicks ($n = 60$) were given an intramuscular injection of 0.5 mg diethylstilbestrol (DES) in 0.5 ml propylene glycol daily for seven days (primary stimulation) and then split into two groups. Following the primary DES treatment there was a period of withdrawal from DES for 12 days in group one (non-primed) birds ($n = 30$) followed by a single injection of progesterone (20 mg/kg) ($n = 10$), oestradiol (10 mg/kg) ($n = 10$) or vehicle (propylene glycol; 1 ml/kg)

($n = 10$). In the second group (primed) the birds ($n = 30$) were re-stimulated daily for two days with DES after a withdrawal period of 10 days followed by a single injection of progesterone ($n = 10$), oestradiol ($n = 10$) or vehicle ($n = 10$) as already detailed for the non-primed birds. All chicks were killed 12–16 h after the single injection; magnum tissue was removed and immediately frozen in liquid nitrogen then stored at -80°C .

All animals were killed in accordance with schedule 1 of the animals (scientific procedures) act 1986, UK under project licence PPL 60/3964.

2.3. RNA preparation

Soft tissues (magnum, shell gland, isthmus, lung, cerebellum, spleen, liver, kidney and embryonic) were homogenised in Lysing matrix D tubes (Qbiogene-Alexis Ltd. Nottingham, UK) containing Ultraspec II total RNA isolation reagent (AMS Biotechnology, Oxon, UK) using a FastPrep FP120 homogeniser (Qbiogene-Alexis Ltd. Nottingham, UK). All other tissues were homogenised in Ultraspec II total RNA isolation reagent (AMS Biotechnology, Oxon, UK) using an Ultraturrax homogeniser (IKA®-Werke GmbH & Co. KG, Germany). Samples were then processed as per the Ultraspec II protocol.

2.4. Transcript determination

2.4.1. PCR and sequencing

1 μg samples of chicken, duck and turkey magnum RNA were reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, UK) according to the manufacturer's protocol. Primers TenpExon1F1 and TenpEnd1 (chicken), D.TenpF3, D.TENP R3, D.TENP F8 and D.TENP R10 (duck) and T.TENPF1 and T.TENPR1 (turkey) ([Table 1](#)) were designed to ensure complete coverage of the *TENP* sequence (see bioinformatics previous analysis). PCR amplification was performed using the following conditions: an initial denaturation at 95°C for 4 min, followed by 40 cycles of 30 s at 95°C , 30 s at 58°C and 30 s at 72°C , followed by an extension of 7 min at 72°C . All products were separated by 2% agarose-gel electrophoresis and visualised using SYBR Safe DNA gel stain (Invitrogen, UK).

The amplified PCR fragments were sequenced with the forward and reverse primers. Sequences were assembled by [Staden \(1996\)](#) to produce consensus sequences.

A phylogram was constructed using Mega5 to infer the evolutionary history of the *TENP* homologues. Included in this analysis was human *LPLUNC2* (NCBI reference sequence no. NM_025227) and chicken *Ovocalyxin 36* a *BPI* like gene (NCBI reference sequence no. NM_001030861) as outliers.

2.4.2. Northern analysis

Magnum RNA (2 μg) and liver RNA (12 μg) were run on a 2% agarose gel under denaturing conditions ([Pelle and Murphy, 1993](#)), SYBR safe DNA gel stain (Invitrogen, UK) was used for visualisation. The RNA was then transferred to an Amersham Hybond N+ nylon membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) after washing the gel in dH_2O for 30 s; 50 mM NaOH, 10 mM NaCl for 45 min; 0.1 M Tris HCl pH7.5 for 45 min; $20\times$ SSC for 60 min and UV-crosslinked (Stratagene Stratalinker) at $120,000\ \mu\text{J}/\text{cm}^2$. A Riboprobe in-vitro Transcription Systems kit (Promega, Southampton, UK) was used to produce a single stranded RNA probe via the T3 RNA polymerase system and incorporated ^{32}P labelled UTP. Template DNA consisted of a *HindIII* linearised pBluescript plasmid (p347_ *TENP*) containing the cloned BPI2 (EMBL HG007958 position 1196–1569) domain present in both potential *TENP* transcripts. Hybridisation of ^{32}P probe was detected using the Typhoon FLA7000 (GE Healthcare Life Sciences, Buckinghamshire, UK).

2.4.3. 5' RACE

5' RACE (Roche Diagnostics 2nd Generation, Mannheim, Germany) was carried out to determine the transcriptional start site of *TENP*.

Briefly, synthesis of first strand cDNA was carried out on magnum RNA using primer SP1 and the mRNA template degraded. cDNA was purified using a High Pure PCR Product Purification kit (Roche Diagnostics, Mannheim, Germany) and polyA tailed at the 3' end. The tailed cDNA was amplified by PCR using the Oligo (dt)-anchor primer provided and a further nested primer SP2. The product from this PCR was run using 3% agarose-gel electrophoresis and visualised using SYBR Safe DNA gel stain (Invitrogen, UK). PCR product was excised from the gel and cleaned from the agarose using a QIAEX II Gel Extraction Kit (Qiagen, UK) and sequenced using primer SP2.

2.5. Reverse transcription quantitative polymerase chain reaction (RT-QPCR)

A first strand cDNA synthesis kit (GE Healthcare Life Sciences, Buckinghamshire, UK) was used for reverse transcription of a 1 µg sample of total RNA as per the manufacturer's instructions. Reverse transcribed samples were diluted by a factor of 10 with H₂O prior to use. [Primer3 \(2013\)](#) was used to design primers TENP RTF and TENP RTR for amplification of chicken TENP; D.TENP F8 and D.TENP R10 for duck TENP. RT-QPCR was carried out with 10 µl of the diluted cDNA and a primer concentration of 20 mM according to Agilent Brilliant II SYBR® Green QPCR master mix (Stratagene, UK) instructions. The following conditions were used for RT-QPCR; 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, 60 °C for 30 s using an MX3000 (Stratagene, UK). Reactions containing no template were run as a control. Standard PCR conditions were used to obtain products for the construction of a standard curve in order to determine the absolute concentration of TENP. PCR products were purified using a QIAEX II Gel Extraction Kit (Qiagen, UK) and quantified using a Nanodrop™ spectrophotometer (Thermo Scientific, UK). Standards were diluted to produce a top standard that was detectable at around 8 cycles during RT-QPCR amplification with six ten-fold serial dilutions forming the standard curve. Products were run on an agarose gel to confirm only products of the correct length with no primer-dimer were amplified as well as ensuring that there was only a single peak dissociation curve. Correct amplification was also confirmed through sequencing of the PCR product. Lamin B-receptor (LBR) expression was measured in the same way to normalise concentrations as used previously ([McDermont et al., 2012](#)). One way or two way ANOVA and least significant difference to test between the means were used as appropriate for statistical analysis of log transformed data. (Genstat 13th edition, VSN International Ltd, Oxon, UK).

2.6. Production and titres of polyclonal anti-TENP antibodies

Production of antibodies was carried out by Dundee Cell Products Ltd, Dundee. Briefly, two rabbits (R106 and R107) were immunised four times at three week intervals by intramuscular injection of synthesised TENP epitopes (AWMDDVLREGVHLPLSH and DAELSIAASNVGLVRRAA) emulsified in Freund's adjuvant. Serum was collected after each immunisation. Antiserum was purified via a two-step affinity purification using cognate peptides coupled to beads.

To measure the titres of anti-TENP in the R106 and R107 antisera, the synthesised epitopes were diluted with 50 mM Na₂CO₃ (pH9.6) to a final concentration of 1 ng/µl (0.5 ng/µl of each epitope) and 50 µl of the solution was added to each well of a 96 well plate. The plate was covered and stored overnight at 4 °C. This was aspirated and the wells incubated for 2 h at room temperature with 200 µl tris-buffered saline (pH7.5), 0.5% tween 20 (TBST), 1% bovine serum albumin (BSA) to block unsaturated binding sites. Pre-immune (null) sera and antisera were serially diluted 1/1000 to 1/32,000 with TBST, 1% BSA, pH7.5. To each well, 10 µl of diluted null sera or antisera were added and the plate incubated for 2 h at room temperature. The plate was washed 5 times with TBST. Horse radish peroxidase (HRP) conjugated Anti-rabbit IgG (SAPU, Edinburgh, Scotland) diluted 1/2000 with TBST, 1%

BSA, pH7.5 100 µl was applied to each well and the plate incubated at room temperature for 2 h. After five washings with TBST, peroxidase activity was detected by adding 100 µl detection solution (100 mM citric acid, 200 mM Na₂HPO₄, O-Phenylenediamine (OPD), H₂O₂). The reaction was stopped with 50 µl 2 M H₂SO₄ and absorbance (490 nm) measured spectrophotometrically.

2.7. Immunohistochemistry (IHC)

Wax embedded tissues were sectioned at 3 µm using a ThermoShandon Finesse microtome, lifted onto vecta slides and incubated at 60 °C for 1 h before they were de-waxed and taken down to water. Each section was then treated with Proteinase K for 20 min at room temperature (antigen retrieval) before loading onto a Dako Autostainer (Dako, Cambridgeshire, UK). A standard IHC protocol was then used; optimal staining was achieved at a 1:3000 dilution of the polyclonal anti-TENP antiserum (107_AWM_1.1) for 30 min. The sections were viewed using a Leica DM 4000 B microscope and images captured using a Leica DC480 camera with Qwin programme for PC (Leica, London, UK).

2.8. Sequencing and database submission

All sequencing was carried out by GATC biotech (Konstanz, Germany) and consensus sequences were submitted to EMBL, chicken TENP (EMBL accession no. HG007958), turkey TENP (EMBL accession no. HG425203) and duck TENP (EMBL accession no. HG425202).

3. Results

3.1. Bioinformatic analysis and transcript confirmation

A BLAT search of the May 2006 chicken genome ([Chicken \(Gallus gallus\) Genome Browser Gateway, 2013](#)) using the published TENP protein sequence (NCBI reference sequence. NM_205026) returned a result indicating a 98.2% identity. However there was a gap in the genomic data between 10248912 and 10249705 bp on chromosome 20. Searching the EST and cDNA database produced the following sequence accession numbers; GenBank accession no. DT657251, BM439389, DT655485, DT655483, DT654774, DT657764, BU357647, BX265690, BX265691, BU210629, BU266397 and AF029841. All sequences were derived from hen reproductive tract except AF029841 which was from the original TENP publication ([Yan and Wang, 1998](#)) that used embryos. These were aligned using [Staden \(1996\)](#) and the consensus sequence produced was used to close the gap in the genomic sequence. The EST and genomic data either side of the gap were identical; the published sequence for TENP was 99.1% identical to the sequence generated by the Staden alignment. When the TENP PCR products generated in this study were sequenced, they were identical to the available genomic and EST sequences. However they differed from the current RefSeq (NM_205026) by an insertion of G between positions 190 and 191 (NM_205026.1:c.190_191insG) and a deletion of G at position 251 (NM_205026.1:c.251delG) resulting in a partial frame shift, altering part of the protein sequence ([Fig. 1](#)). Analysis of the sequence from this study (EMBL HG007958) identified a potential alternative translational start site ([Fig. 1](#)). The published translational start site ([Yan and Wang, 1998](#)) at genome position Chr20:10,647,277 (reverse strand) of the November 2011 build with the signal peptide prediction MGALLALLDPVQPTRA gives a signal peptide probability of 0.661 and a max cleavage site probability of 0.651 whilst the new putative translational start site at position Chr20:10,647,544 (reverse strand) in the November 2011 build identified in this study results in a signal peptide prediction of MGTANRKGSAPVPLCTMGALLALLDPVQP that gives a stronger prediction for the signal peptide probability (0.996) and a maximum cleavage site probability of 0.534. 5' RACE using magnum RNA supported the presence of the transcript postulated in this paper,

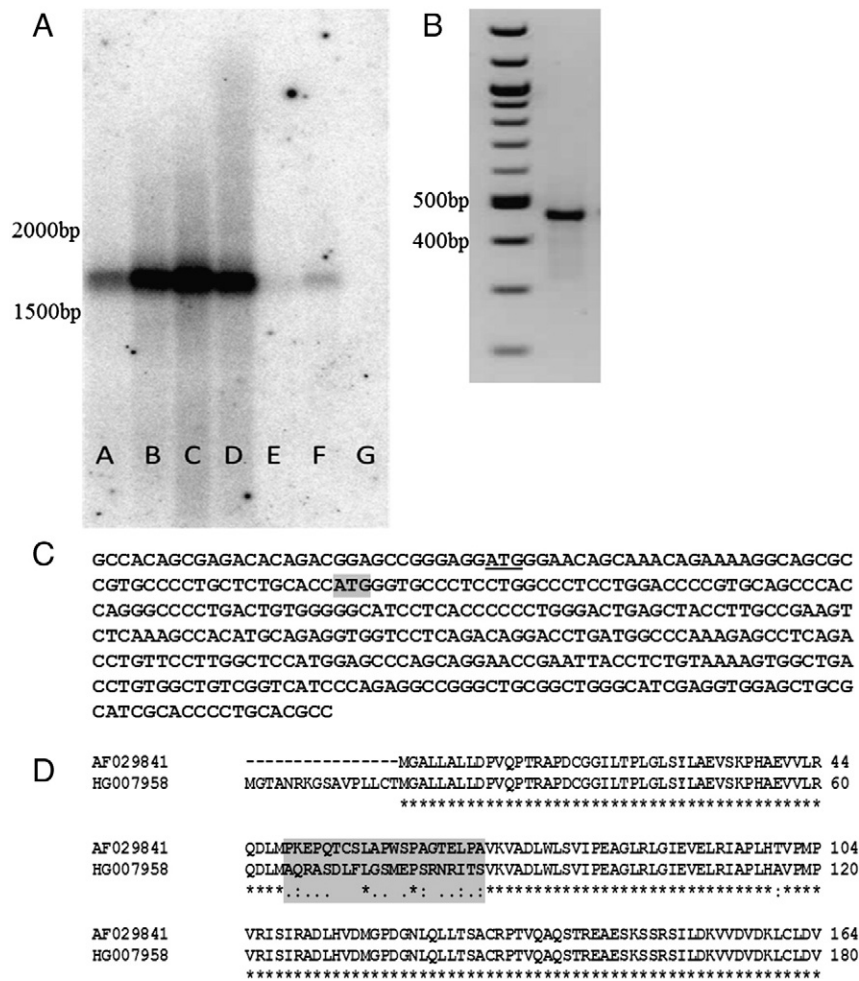


Fig. 1. Northern analysis and 5' RACE. A) Northern analysis of p347_TENP. Lanes A–C contain 2 µg broiler magnum RNA, lanes D–F contain 2 µg layer magnum RNA, 12 µg liver RNA (G) is used as a negative control. B) 5' RACE using primer SP2 resulting in one band. C) cDNA sequencing of the 5' RACE product from B confirmed the presence of an alternative translational start site (underlined), the published start site is highlighted (■). D) The first 180aa of the new putative protein (HG007958) translated from the alternative start site in C is shown in a clustal alignment with the published TENP sequence (AF029841), the location of the frameshift is highlighted (■). The proposed new protein has 455 amino acids.

which in turn supported the new translational start site (Fig. 1). No evidence was found for a sequence which would support the previously published translational start site, however this may be expressed below the detection level of the methods used (Fig. 1). The same method was used to detect the transcriptional start site used in embryonic brain tissue (not shown) however no transcript was detected.

It was noted that the EST data, although supporting the presence of each BPI-like domain in the reproductive tract, offered little evidence for their expression in one full length TENP transcript, rather supporting that the protein may be expressed as two separate molecules each encoding for one of the BPI domains (Fig. 2). PCR using magnum cDNA with primers TENP Exon1F1 and TENP End1 designed to amplify

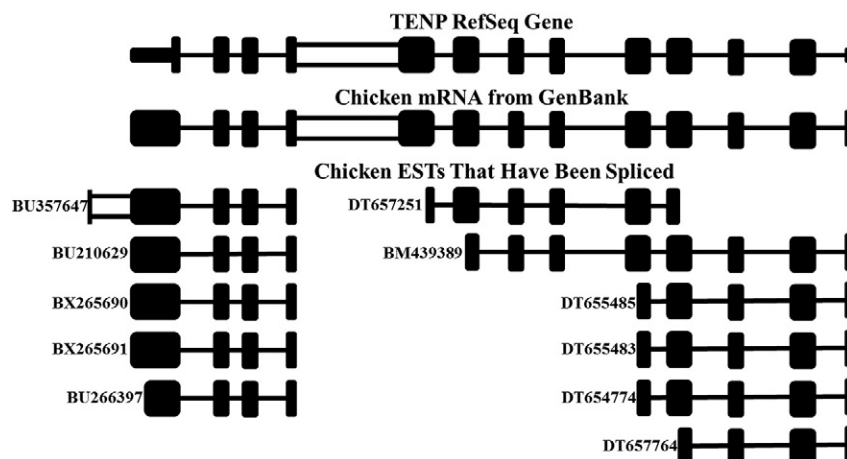


Fig. 2. Schematic representation of chicken ESTs using the EST data displayed by the UCSC Genome Browser website. ESTs were all isolated from the reproductive tract of adult hens and provided no evidence for a transcript where both BPI-like domains are expressed in one molecule.

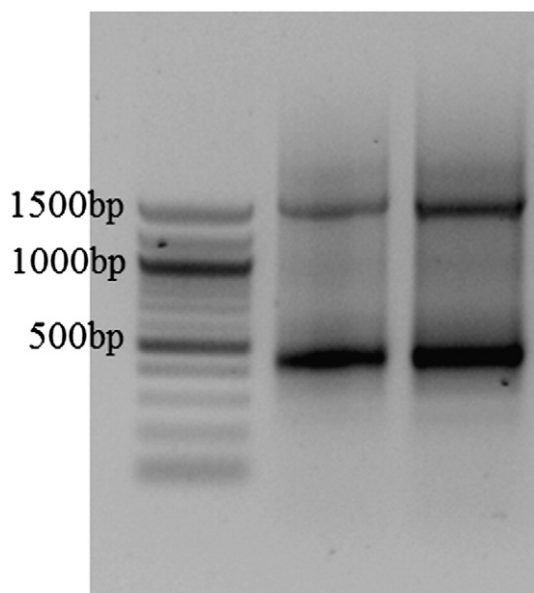


Fig. 3. *TENP* PCR — Amplification of *TENP* from magnum cDNA using primers *TENP* Exon1F1 and *TENP* End 1 in the first and last exon of *TENP*. Lane 1, 100 bp molecular weight marker; lanes 2 and 3 *TENP*. Results indicate two *TENP* transcripts with sizes corresponding to a transcript encoding the full *TENP* protein and also that of just one BPI domain.

the whole of the *TENP* protein coding region from chr20:10642981 to 10647548 resulted in two strong bands. Sequencing of these PCR products confirmed that one of the transcripts encoded for a full length transcript including the new predicted translational start site, the second smaller transcript would, if translated, encode only the BPI2 region of the *TENP* protein (Fig. 2). In order to confirm the expression of these *TENP* transcripts in magnum tissue northern analysis using an RNA probe corresponding to the BPI2 domain, present in both forms detected by PCR, was carried out. The results proved the presence of only the full length transcript in both layer and broiler type birds (Fig. 1) but showed no indication of a smaller transcript despite the PCR evidence. In general the intensity of the signal from the layer line was lower and more variable than that of the broiler (Fig. 1). It was predicted that chicken *TENP* has 16 exons (Table 2).

Potential *TENP* homologues were identified in both duck and turkey genomes and their expression was confirmed using PCR and sequencing. The phylogenetic analysis (Fig. 4) suggests that *TENP* molecules from avian species are more similar to each other than either *Ovocalycin* 36 or *LPLUNC2*.

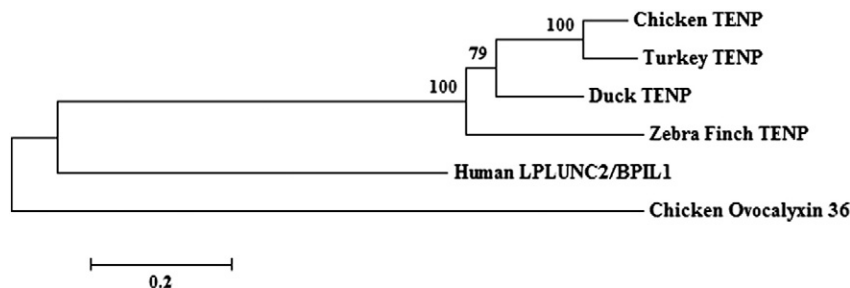


Fig. 4. Evolutionary relationships of avian *TENP* homologues, *LPLUNC2* and *Ovocalycin* 36. A phylogram indicating the evolutionary history of *TENP* was inferred using the Neighbour-Joining method (Saitou and Nei, 1987) from an alignment of the mature proteins. Included for comparison were human *LPLUNC2* and chicken *Ovocalycin* 36. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The branch lengths are proportional to the evolutionary distances which were computed using the Poisson correction method (Zuckerandl and Pauling, 1965). The units are the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The common name of the species is included with the protein names.

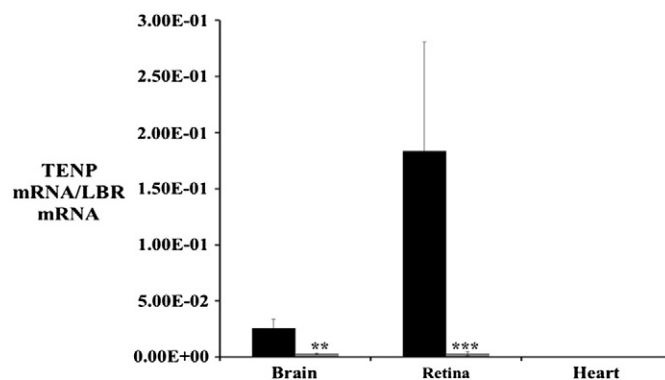


Fig. 5. Expression of *TENP* mRNA in embryonic days 10 (■) and 16 (▒) chicken brain, retina and heart tissues measured by RT-QPCR (n = 6). *TENP* expression was corrected for LBR expression to normalise for any differences between tissues. ANOVA indicated that embryonic stage was significant at $P < 0.001$ and tissue was significant at $P < 0.001$. *TENP* expression clearly diminished with development. Heart was included as a negative control, as expected no expression was detected. Significance between embryonic stage within tissue is indicated $P < 0.001$ (***) and $P < 0.01$ (**).

3.2. Tissue expression

TENP expression was detected in embryonic brain and retinal tissues (Fig. 5) and decreased as development progressed from E10 to E16. In contrast measurement of *TENP* expression in adults indicated that expression was restricted to the oviduct of adult hens and almost exclusively the magnum with no detectable expression in either the brain or retina suggesting *TENP* plays a different role in the adult bird (Fig. 6). Expression was also restricted to the magnum of the oviduct in the duck tissues sampled (Fig. 6). *TENP* expression was measured in the magnum of the oviduct when the egg was either in the magnum, the shell gland or during a pause day. Eggs in the shell gland were classified as either early, mid or late depending on the level of calcification as determined by electron microscopy. The position of the egg in the oviduct or the occurrence of a pause day, when the hen did not ovulate and so no egg was present, did not significantly alter the level of *TENP* expression in the magnum (data not shown) (ANOVA, $P = 0.083$). However if the data was categorised into when an egg was in the magnum or had just recently left it (early) versus the later stages of calcification (mid/late) then the difference is verging on significance (ANOVA, $P = 0.051$). The level of *TENP* expression in both the magnum and the shell gland was higher ($P < 0.001$) in birds in-lay than in adult hens whose oviduct had regressed due to incubation behaviour (Fig. 7) which suggests that the level of *TENP* expression is affected by the reduction in gonadotrophics which in turn causes reduction in

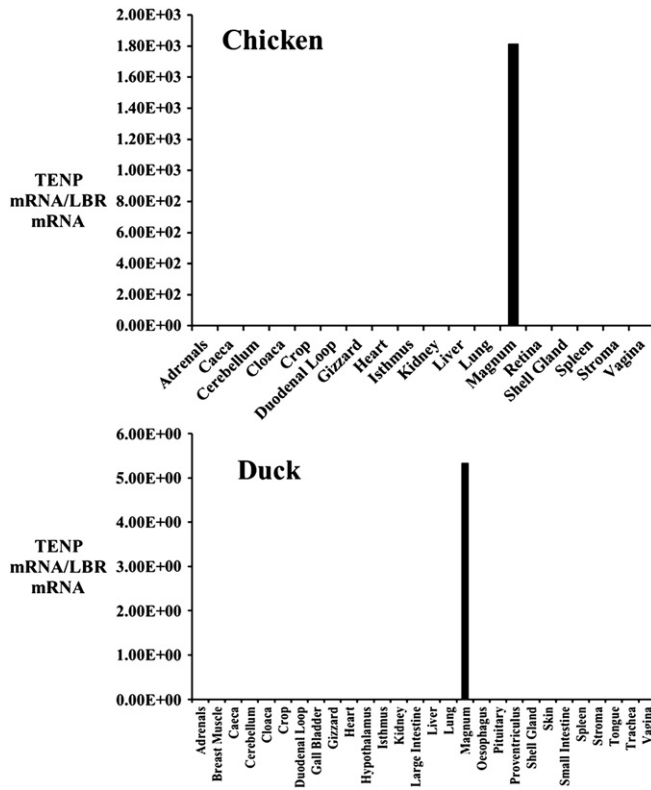


Fig. 6. Expression of *TENP* mRNA in a range of adult chicken ($n = 4$) and duck ($n = 3$) tissues measured by RT-QPCR. *TENP* expression was corrected for chicken LBR and duck LBR expression to normalise for any differences between tissues. Expression is restricted to the oviduct in both species with the greatest level of expression seen in the magnum.

steroid secretion from the ovary. Oestrogen is well characterised as a key regulator in the development and function of the oviduct and is therefore unsurprisingly linked to regulation of the expression of many egg specific genes. When oestrogen and progesterone were administered to juvenile hens, *TENP* expression (Fig. 8) was higher in birds treated with the two steroids ($P < 0.001$) and where priming with an oestrogenic compound had been performed overall expression increased ($P < 0.001$).

3.3. Immunohistochemistry

The anti-TENP antiserum (107_AWM_1.1) produced positive staining in the tubular gland cells of the magnum (Fig. 9A). The ciliated

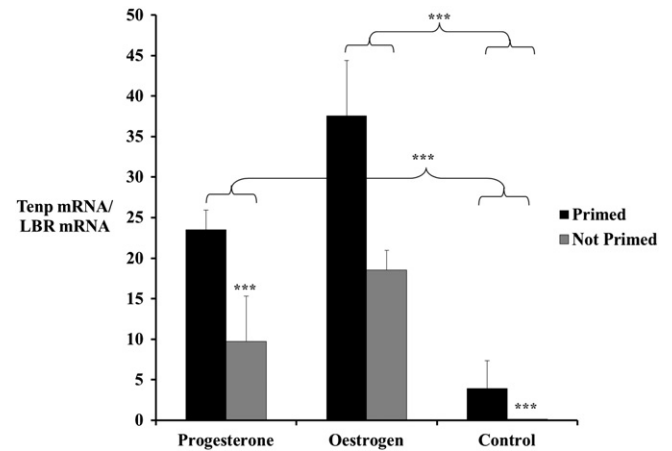


Fig. 8. Expression of *TENP* mRNA in the magnum of juvenile chicks treated with steroids measured by RT-QPCR ($n = 10$, mean \pm SEM). *TENP* expression was corrected using LBR expression. Female chicks at 3 weeks of age were either primed with diethylstilbestrol (■) or vehicle (□) then subsequently treated with either progesterone, oestradiol (oestrogen) or vehicle (control). ANOVA indicated primed or not primed was significant at $P < 0.001$; steroid treatment was significant at $P < 0.001$. Significance between primed state within treatment is indicated, $P < 0.001$ (***) and significance between treatments regardless of primed state between the brackets, $P < 0.001$ (***)

and non-ciliated cells lining the magnum region of the oviduct did not react to the primary antibody. No staining was observed in the isthmus, shell gland or caecum (Fig. 9C–E).

4. Discussion

The conservation of TENP among avian species and the results outlined in this study strongly suggest a major egg specific role for TENP protein in the adult hen. However, local tissue activity cannot be ruled out and TENP may provide antimicrobial protection in both the egg and the oviduct. The fact that this protein also plays a transient role in the developmental stages of the chicken embryo suggests a dual purpose for TENP. This makes understanding how expression of *TENP* is controlled and of course its purpose extremely valuable in providing an insight into the biology of not only the egg but the chick as well.

The EST data aligned to the May 2006 chicken genome assembly confirmed the expression of both of the *TENP* BPI domains in the hen reproductive tract but offered little evidence for their expression in one full length *TENP* transcript, as originally identified in neural precursors (Chicken (*Gallus gallus*) Genome Browser Gateway, 2013), rather supporting that the protein may be expressed as two separate

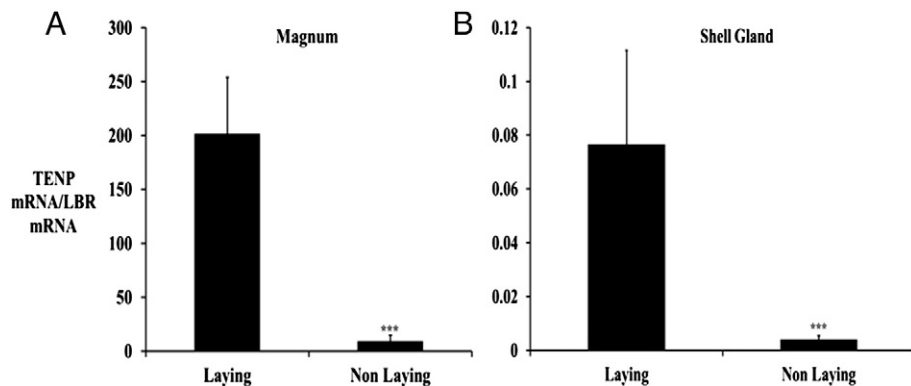


Fig. 7. Expression of *TENP* mRNA in A) magnum and B) shell gland tissue of laying (L) and out of lay (NL) birds measured by RT-QPCR ($n = 11$, mean \pm sem). Non-laying hens were those where the oviduct had regressed due to the withdrawal of gonatrophic support with the onset of incubation behaviour. *TENP* expression was corrected using LBR expression. Note the large difference in the Y-axis scale between A and B. Significance between laying state is indicated at $P < 0.001$, ***.

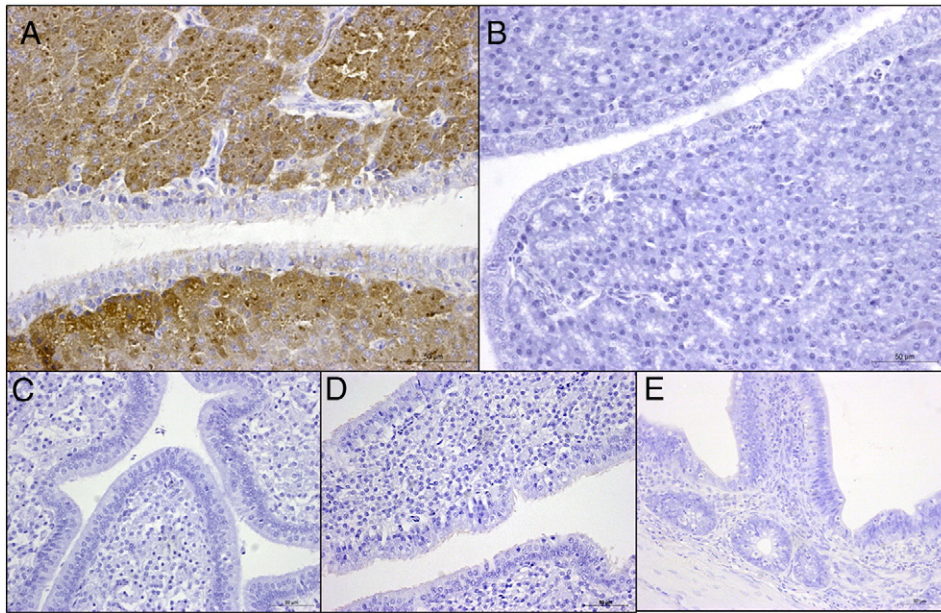


Fig. 9. The tubular gland cells of the magnum region (A) of the oviduct stained positive and the surface epithelium (ciliated and non-ciliated cells) did not stain with anti-TENP antisera (107_AWM_1.1). The corresponding negative controls for A are shown in image B. The isthmus (C), shell gland (D) and caecum (E) were not reactive to the primary antibody.

molecules each encoding for one of the BPI domains (Fig. 3). Sequencing of PCR products using magnum cDNA with primers designed to amplify the whole of the *TENP* protein coding region produced a full length transcript including the new predicted translational start site and a second smaller transcript which if translated would encode only for the BPI2 region of the *TENP* protein (Fig. 2). Although in theory intensities of DNA bands on a gel amplified from targets with the same primers should be indicative of the relative abundance of each transcript in the starting sample if amplification efficiency is equal (Cottrez et al., 1994), factors such as amplicon size or sequence can lead to a bias in amplification with smaller amplicons being amplified more efficiently (Cha and Thilly, 1993) or possibly differences in reverse transcription efficiency. Indeed Northern analysis indicated that the full length transcript (Fig. 1) was the dominant form of *TENP* and the lack of signal for the smaller transcript suggested that it may be below the threshold of detection for this method contrary to the PCR results. Although these results indicate that a transcript encoding both BPI-like domains is the dominant form in the oviduct the presence of a smaller transcript cannot be ruled out completely. It may be that this gene encodes for alternate forms of *TENP* where only one of the BPI domains is expressed, as seen with SPLUNCs (Bingle and Craven, 2002).

The PCR products from this study (*TENP* Exon1F1-Tenp End1) were identical to the available genomic and EST sequences yet contained an indel when compared to the previously published *TENP* sequence resulting in a frameshift (Fig. 1). We would therefore propose that the previously published protein sequence for *TENP* is either incorrect or specific to the strain used in that study. Potential *TENP* homologues were identified in both the duck and turkey genomes and sequencing of PCR products confirmed the presence of *TENP* mRNA in the magnum of both species. A potential *TENP* homolog is predicted in the zebra finch (NCBI Reference Sequence: XP_002192628.1) and *TENP* has also been identified in proteomic analysis of emu egg white (Maehashi et al., 2010). These results coupled with a high level of sequence similarity suggest that *TENP* is highly conserved across avian species. From the phylogenetic analysis (Fig. 4) the *TENP* molecules are more similar to each other than either *Ovocalycin 36* or *LPLUNC2*. The analysis infers that *TENP* is a divergent ortholog of *LPLUNC2*, and that *Ovocalycin 36* may have evolved from an ancestral *LPLUNC2*-related gene as previously suggested (Chiang et al., 2011), perhaps from a duplication event occurring before mammals and birds diverged.

Since this analysis was carried out, deposits in the GenBank database made by Kinoshita K., et al. became visible (accession no. BAM13270.1, BAM13271.1, BAM13272.1, BAM13273.1, BAM13274.1 and BAM13275.1). The text proposed that *TENP* is identical to the chicken egg white protein ovoglobulinG2 and is expressed as a full length protein in the adult reproductive tract which would concur with the results seen in this study, however as far as we are aware, no paper has been published related to this observation. We believe that these submissions refer to a globulin fraction separated by Longworth et al. (1940). In the paper Longworth et al. identified three globulins G₁, G₂ and G₃ using electrophoresis to separate egg white and examining the electrophoretic patterns of ovalbumin and the globulin fractions.

Analysis of *TENP* sequence from the genome discovered a potential alternative translational start site which was confirmed as the dominant site used for transcription in magnum tissue. Because the expression of *TENP* is low in the embryo we were unable to confirm the transcriptional start site. The proof from the magnum that the predominant translational start site is at position 32 (Fig. 1C) combined with the Signal P predictions probability score of 0.996 for the new signal peptide probability and the signal peptide cleavage point suggests that at least in the adult the sequence proposed in our paper is correct. There is no experimental evidence to support the translational start site, however, if we were to assume a typical Kozac initiation of translation (Kozak, 1999) then the evidence for the transcription start site from the 5' RACE makes the proposed translational start site the most likely. However it must be noted that in the absence of functional evidence the translational start site cannot be confirmed as upstream ATGs occur frequently in vertebrate mRNA (Rogozin et al., 2001) and we cannot rule out the possibility of multiple sites of translation (Kochetov et al., 2005).

Measurement of *TENP* expression in embryonic brain and retinal tissues concur with the developmental down-regulation seen by Yan and Wang (1998); expression seen at E10 is dramatically decreased by E16 and no expression is detected in adult brain or retinal tissues (Fig. 5). In contrast, expression of *TENP* in the adult hen is restricted to the oviduct (Fig. 6) suggesting that at this stage *TENP* plays a different role. Expression is almost exclusively in the magnum with expression here around 400× more than in the shell gland and almost 10,000× greater than in embryonic retina tissue. The magnum is responsible for the production of egg white and the high level of expression here would suggest that *TENP* is primarily a component of egg white.

However this study did detect some expression in other regions of the oviduct which would concur with the proteomic evidence for TENP in the other egg compartments e.g. the shell. Immunohistochemistry confirmed the presence of TENP as a protein in the tubular gland cells of the magnum region of the oviduct (Fig. 9); this would support the secretion of TENP. Interestingly TENP was not detected in the epithelial cells which may indicate that TENP is less involved in local protection of the oviduct. In duck tissues *TENP* expression is also restricted to the magnum of the oviduct (Fig. 6). *TENP* expression in the caecum had previously been reported by Chiang et al. (2011) however when using our quantitative approach no significant expression was measured in this tissue compared to other tissues (Fig. 6) which was further confirmed through IHC (Fig. 9).

Expression in the magnum of the oviduct did not differ significantly in relation to the position of the egg in the oviduct at the time of sampling or if it was a pause day when no egg was present, although there was a tendency for the level to be lower when the egg was in the magnum or had recently left it ($P = 0.051$) (data not shown). This pattern of expression is typical of egg proteins and has been described previously for others including gallin (Gong et al., 2010) and ovalbumin (Muramatsu et al., 1994). In the magnum and shell gland there is significantly more *TENP* expression when the hen is in-lay compared to when the oviduct is regressed (Fig. 7) suggesting that the expression of *TENP* is specifically up-regulated during egg production when steroids are elevated and is likely to be under the control of gonadal steroids. This was confirmed by the measurement of *TENP* expression after the administration of oestrogen and progesterone to juvenile hens, with the increase in expression greatest with the dose of oestrogen compared to that of progesterone. Most importantly the response was strongest when the animals had previously been primed with an oestrogenic compound and showing that oestrogen and progesterone act synergistically as one would expect of an oviduct gene controlled directly or indirectly by gonadal steroids. Analysis for transcriptional factor binding sites 20 kb upstream of the gene using MATCH (2013) did not identify any oestrogen or progesterone receptors so the effect may be secondary to stimulation of transcription factors by these steroids.

These results strongly suggest that in the adult hen *TENP*'s role is as a major component of the egg. Although local tissue activity, for example antimicrobial protection of the oviduct cannot be ruled out and it may serve both these roles. It is also notable that proteomic analysis has recently identified *TENP* as a possible requisite host protein in infectious bronchitis virus (IBV) life cycles (Kong et al., 2010). The relationship of *TENP* to the PLUNC/BPI family of proteins suggests a role for *TENP* in the innate defence of eggs against pathogens and the presence of LPS binding domains would make an antimicrobial role seem possible as demonstrated with Ovocalyxin-36 (Gautron et al., 2011). If this is the case it is interesting to note that *TENP* is present in all components of the egg and is a significant egg white component representing ~0.1–0.5% of the total protein, similar to the levels of ovoinhibitor, a multitype serine proteinase inhibitor which is present at 0.1–1.5% (Guerin-Dubiard et al., 2006). The large number of known antimicrobial proteins already identified in the egg would make this seem plausible although experimental proof of the activity of *TENP* does not yet exist.

5. Conclusion

TENP, a PLUNC homolog and member of the BPI family of LPS binding proteins is expressed in the retina and brain of chicken embryos. Expression diminishes over time in the embryo but in any case it is 10,000 times less than in adult oviduct. In the adult hen and duck *TENP* expression is restricted to the oviduct, almost exclusively the magnum. The expression of *TENP* is up-regulated in laying hens versus those with a regressed oviduct suggesting it to be under the control of gonadal steroids; this is further supported by the increased expression of *TENP* after administration of oestrogen and progesterone in juveniles.

In adult birds *TENP* shows the classic signature of an egg specific gene. This, coupled with its relationship to the BPI/PLUNC family, suggests a role in protecting the egg from microbial attack.

Authors' contributions

NW carried out the sequence and expression analysis, antibody titre measurement and analysis of data. MMB and LS carried out immunohistochemistry. NW, ICD, PWW and MMB carried out the sample collection and experimentation. NW and ICD carried out the bioinformatics and phylogeny analysis. ICD supervised and obtained the funding for the CASE Studentship. All authors contributed and approved the final manuscript.

Conflict of interest statement

The authors declare no conflicts of interest.

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